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THE INTERACTION OF VIRAL RNA WITH
CULTURED MAMMALIAN CELLS

by



DOROTHY RAE TOVELL

A THESIS

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The undersigned certify that they have read,
and recommend to the Faculty of Graduate Studies for
acceptance, a thesis entitled "THE INTERACTION OF
VIRAL RNA WITH CULTURED MAMMALIAN CELLS", submitted
by Dorothy Rae Tovell in partial fulfilment of the
requirements for the degree of Doctor of Philosophy.

ABSTRACT

DMSO and DEAE-D have been shown to stimulate the formation of infectious centers when added to media in which L cells and Mengo RNA are incubated. Maximum stimulation is obtained with DMSO when it is present at a concentration of 10-12.5% in 0.6 M sucrose/PBS, while DEAE-D is maximally effective at a concentration of 100 μ g/ml in PBS. Putrescine or polyornithine did not stimulate infectious center formation significantly.

A comparative study, designed to define the optimal conditions for infecting L, HeLa, and human diploid cells with Mengo RNA, showed that the cell types differ with respect to the concentrations of sucrose in both PBS and PBS containing 10% DMSO that produce maximum stimulation of infectious center formation. With cells of all three types, DEAE-D was most effective when present at a concentration of 100 μ g/ml in PBS. Direct comparisons of infectious center formation in these cell types using the sucrose, sucrose-DMSO, and PBS-DEAE-D media optimal for each, revealed that the best medium for the titration of viral RNA in L and HeLa cells is PBS-DEAE-D, while in diploid cells the sucrose-DMSO medium is best. No correlation was found between the ability of cells to retain viability in the various media and the number of infectious centers formed therein.

The uptake of homogeneous, ^3H -labelled Mengo RNA by L cells from PBS, 0.6 M sucrose/PBS, 0.6 M sucrose/PBS-10% DMSO, and PBS containing 100 μ g DEAE-D/ml was measured. It was shown that extremely small quantities of RNA are

taken up by cells incubated in sucrose/PBS, that the uptake is not increased by the addition of DMSO, and that less RNA is taken up from either sucrose medium than from PBS. DEAE-D produces a striking increase in the amount of RNA that becomes cell-associated, but most of this RNA does not penetrate the cell membrane. Attempts to isolate significant amounts of undegraded (35S) RNA from cells incubated with ³H-Mengo RNA in any of the media were unsuccessful, suggesting that the survival of an intact RNA molecule at the intracellular level may be a very rare event.

Evidence is presented to support the premise that some aspect of cell-RNA interaction is the limiting factor in the assay of viral RNA, and, with suspended cells at least, only a fraction of the potentially infectious RNA is detected. Significant amounts of infectious, undegraded RNA can be recovered from media in which cells have been incubated.

Data presented herein support the proposition that in the PBS-DEAE-D medium, viral RNA is taken up by cells in the form of a complex with the polycation, and it seems likely that it must be released therefrom in order to express its genetic information. The mechanism by which DMSO stimulates the demonstrable infectivity of viral RNA is not clear, but it would appear that it somehow does increase the efficiency with which the RNA enters into polyribosome structures.

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LIST OF ABBREVIATIONS

BPA	bovine plasma albumin
DEAE-D	dimethylaminoethyl dextran
DMSO	dimethylsulfoxide
EDTA	ethylene diamine tetraacetic acid (sodium salt)
PBS	phosphate-buffered saline
RNA	ribonucleate
RNase	ribonuclease
TCA	trichloroacetic acid
TRIS	tris(hydroxymethyl)-amino methane
S	intrinsic sedimentation coefficient
pfu	plaque-forming unit
cpm	counts per minute
rpm	revolutions per minute

All temperatures are expressed as degrees Centigrade.

INTRODUCTION

The unique importance of the nucleic acid in the reproduction and genetic continuity of RNA-containing viruses was first demonstrated directly by Gierer and Schramm in 1956, although the corresponding central role played by bacteriophage DNA had been recognized four years earlier (Hershey and Chase, 1952). It was shown that RNA isolated from tobacco mosaic virus was capable of initiating an infection in tobacco leaves leading to the formation of virus particles identical to those from which the nucleic acid was isolated (Gierer and Schramm, 1956). The same report included the first description of the phenol method for the isolation of infectious viral RNA. This relatively quick and simple method was shown to remove all the protein and to give essentially quantitative recovery of high molecular weight RNA. Shortly after the publication of the findings of Gierer and Schramm, the phenol technique was applied to the isolation of RNA from both mammalian tissues (Colter and Brown, 1956) and a mammalian virus (Colter, Bird and Brown, 1957). The latter communication, describing the isolation of an infectious RNA from Mengo encephalomyelitis virus, was only the first of numerous reports to describe the extraction of infectious RNA from a mammalian virus. Table I.1 lists those mammalian viruses from which infectious RNA has been isolated, and indicates the systems which have been employed to demonstrate the

Table I.1

Mammalian viruses from which infectious RNA has been isolated^a

Virus	Assay System	Investigators
Mengo encephalomyelitis	Mice (intracerebral)	Colter, Bird & Brown (1957)
	Mice (intracerebral)	Colter, Bird, Moyer & Brown (1957)
West Nile encephalitis	Tissue culture (L cells)	Ellem & Colter (1960a,b)
	Mice (intracerebral)	Colter, Bird & Brown (1957)
Poliomyelitis	Mice (intracerebral)	Colter, Bird & Brown (1957)
	Tissue culture (HeLa cells)	Alexander <u>et al.</u> (1958)
	Tissue culture (HeLa cells)	Schäffer & Mattern (1959)
	Tissue culture (HeLa, monkey kidney)	Holland <u>et al.</u> (1959a,b)
Semliki forest encephalitis	Mice (intracerebral)	Cheng (1958)
Eastern equine encephalitis	Embryonated eggs (allantoic cavity)	Wecker & Schäfer (1957)
Western equine encephalitis	Embryonated eggs (allantoic cavity), chick embryo fibroblast cells	Wecker (1959, 1960)
Mouse encephalomyelitis	Mice (intracerebral)	Franklin <u>et al.</u> (1959)
Encephalomyocarditis	Krebs 2 ascites tumor cells	Huppert & Sanders (1958)
	Mice (intracerebral)	Liebenow & Schmidt (1959)
Murray Valley encephalitis	Chick embryos (chorio-allantoic membrane)	Ada & Anderson (1959a)

	Chick embryos (chorio-allantoic membrane)	Anderson & Ada (1959)
Dengue I and II	Mice (intracerebral)	Ada & Anderson (1959b)
Theilers GD VII	Mice (intracerebral)	Ada & Anderson (1959b)
Foot-and-mouth disease	Pig kidney cells, mice (intramuscular)	Brown <u>et al.</u> (1958)
	Bovine kidney cell cultures	Bachrach (1960)
	Mice (intracerebral)	Mussgay <u>et al.</u> (1958)
	Guinea pig	Spuhler (1959)
Tick-borne encephalitis	Mice (intracerebral)	Sokol <u>et al.</u> (1959)
Coxsackie A-7, B-4, B-5	HeLa and human amnion cell cultures	Sprunt <u>et al.</u> (1959)
Coxsackie A-9, B-1	Tissue culture (HeLa, human amnion, etc.)	Holland <u>et al.</u> (1959b)
ECHO 1 and 8	HeLa and human amnion cell cultures	Sprunt <u>et al.</u> (1959)
ECHO 8	Tissue culture (HeLa, human amnion, etc.)	Holland <u>et al.</u> (1959b)

^a Adapted from Colter & Ellem (1961a)

infectious nature of the preparations. The phenol method as described by Gierer and Schramm was used for the isolation of RNA in most of the studies listed in the table, but other methods, as well as modifications of the original technique, have been used successfully with certain viruses. Wecker (1959) reported that the RNA of mature Western equine encephalitis (WEE) virus particles was extractable in an infectious form by hot, but not cold, phenol. Deoxycholate has been used to liberate an infectious product from Eastern equine encephalitis (EEE) virus (Richter and Wecker, 1963) and also from Murray Valley encephalitis virus (Anderson and Ada, 1959), while a phenol-detergent combination was used to release the RNA from foot-and-mouth disease (FMD) virus (Bachrach, 1960). The infectious nucleic acid can be dissociated from the protein of some viruses by very simple means. For example, incubation of poliovirus in hot hypertonic saline (Schaffer and Mattern, 1959), and either heating or acidification to pH 5 of FMD virus (Bachrach, 1961; Brown and Cartwright, 1961) were shown to result in the release of infectious RNA from the virus particles.

As Table I.1 suggests, infectious RNA has been isolated by many investigators from many different viruses, so that in most cases it is now a routine procedure. With respect to the myxovirus class, however, unequivocal evidence that an infectious RNA has been isolated has not yet been presented. There have been a number of reports dealing with the failure of attempts to isolate biologically active RNA

from influenza virus (Sokol and Szurman, 1959; Ada et al., 1959), from Newcastle disease virus (NDV) (Ada et al., 1959; Benedict et al., 1960) and from fowl plague virus (Schäfer, 1959). On the other hand, other workers have reported that they have obtained positive results with influenza virus (Maassab, 1959, 1963; Portocalà et al., 1959), NDV (Portocalà et al., 1959) and with Ranikhet Disease virus (Dhar et al., 1963). However, the progeny resulting from some of these alleged RNA-initiated infections were reported to differ from the original virus preparation (from which the RNA was isolated) with respect to host range (Maassab, 1963) or antigenic composition (Portocalà et al., 1959). These and other observations are difficult to reconcile with the observations of other investigators that the biological properties of a virus are retained by the progeny resulting from an infection produced by RNA from that virus (e.g., Gerber and Kirschstein, 1960).

With very few exceptions (Thely et al., 1961), viral RNA preparations have exhibited a very low degree of infectivity compared with that of the virus suspensions from which they have been prepared. Their activity has been of the order of 0.1 percent that of the corresponding virus suspensions. Various criteria have been used to demonstrate that the infectivity is due to the nucleic acid, and not to trace amounts of intact virus. For example, the activity of RNA preparations has been shown

to be abolished by brief exposure to low levels of ribonuclease, an enzyme to which the corresponding viruses are resistant. Also, purified, specific, immune gamma globulins have been shown to neutralize the infectivity of intact virus particles but not that of the corresponding RNA. Both anti-viral and normal serum have been shown to inactivate RNA, due, in all probability, to the presence of ribonucleases in the serum. In addition, the active principle in infectious viral RNA preparations sediments much less rapidly than does intact virus in the ultracentrifuge.

To account for the low levels of infectivity of RNA preparations, it has been suggested that only a small fraction of the RNA molecules survive the extraction procedure in an intact form. Henry and Younger (1963) have presented data, obtained from studies with RNA isolated from poliovirus, which suggest that the groups necessary for infection are distributed throughout a single polynucleotide chain, and that a single break at any point in the molecule abolishes infectivity. Colter et al. (1957) showed, by ultracentrifugal analysis, that RNA isolated from virus-infected tissues contained two optically detectible components (now known to be ribosomal in origin) with sedimentation velocities of approximately 16 and 32S, and that the infectious component seemed to travel with the faster sedimenting species. On the basis of more

precise measurements, using infectivity as the marker, infectious RNA's of FMD virus (Strohmaier and Mussgay, 1959) and encephalomyocarditis (EMC) virus (Burness et al., 1963) were estimated to have a sedimentation velocity of 37S. These results confirmed the suggestion that infectious RNA molecules (from any particular virus at least) are of a uniform size, but provided no information regarding the proportion of molecules in a given RNA preparation which are potentially infectious. Montagnier and Sanders (1963) examined RNA isolated from a purified preparation of P³²-labelled EMC virus by rate zonal centrifugation on sucrose density gradients. The sedimentation of the viral RNA was followed by infectivity and radioactivity measurements, while a large excess of cellular RNA, added as a marker, was detected optically. Radioactivity and infectivity were found to sediment in a single peak ahead of the 30S peak of ribosomal RNA. Very little radioactivity and no infectivity were found to sediment behind this peak. These results demonstrated that, in the case of this particular virus at least, the viral RNA released by phenol extraction is homogeneous with respect to size, and that the infectivity resides only in these molecules. By comparing the sedimentation velocity of the viral RNA (or more precisely, the molecular weight calculated from the sedimentation velocity) and analytical data on the RNA content of virus particles,

these investigators demonstrated that an infectious molecule corresponds to the entire RNA content of one virus particle.

It has been suggested that viral RNA solutions, when carefully prepared, may contain as many potentially infectious units as do the corresponding virus suspensions, and that the failure to detect them simply reflects the limitations of the assay systems used (Ellem and Colter, 1961a). This premise has received some support from the fact that certain modified assay procedures have been reported to give infectious RNA titers 10^4 - 10^5 times greater than could be obtained by the assay systems in use ten years ago. In fact, Bachrach (1966) claims to have obtained more infectious units in an RNA preparation than were measured in the original virus suspension.

Alexander et al. (1958) introduced the first modification of the standard assay procedure. These investigators found that when viral RNA preparations were diluted in hypertonic salt solutions their demonstrable infectivities were much greater than when they were diluted in solutions of physiological ionic strength. Subsequently, many investigators have shown that, with respect to both the number of plaques produced and the reproducibility of the assay, diluting the viral RNA in either hypertonic salt solutions or solutions of elevated osmolarity is preferable to titrating the RNA in solutions of physiological ionic

Table I.2

Assay systems for infectious viral RNA

RNA	Assay cells	RNA Diluent	Investigators
Polio	HeLa and human amnion monolayers	1M NaCl, pH 7.2	Alexander <u>et al.</u> (1958)
Polio	Human amnion monolayers	1M NaCl, pH 8.2	Koch <u>et al.</u> (1960)
Japanese B encephalitis	Chick embryo monolayers washed with 1M NaCl	1M NaCl, pH 7.6 - 8.0	Nakamura and Ueno (1963)
EEE	Chick embryo monolayers washed with 1M NaCl	1M NaCl, pH 8.2	Colón and Idoine (1964)
Polio	HeLa monolayers	2M MgSO ₄ , pH 7.2	Holland <u>et al.</u> (1960)
Coxsackie B1	BHK monolayers, final wash 1M NaCl	2M MgSO ₄	Crick <u>et al.</u> (1966)
FMD			
EEE	Chick monolayers	MgSO ₄	Mayer and Sokol (1961)
Polio	Primary monkey kidney monolayers	1M MgSO ₄	Wecker <u>et al.</u> (1962)
Polio	HeLa in suspension	2M MgSO ₄	Schaffer (1962)
Polio	HeLa in suspension	0.9 M NaCl	Ellem and Colter (1961a)
Mengo	L in suspension	0.7 M Sucrose	Ellem and Colter (1960a)
EMC	Krebs II ascites in suspension swollen in 0.001 M phosphate buffer	1.3 M Sucrose	Montagnier and Sanders (1962)

strength. A number of the cell-RNA systems in which this has been shown to be the case are listed in Table I.2. Also shown are the incubation media that the various investigators found to be optimal for the demonstration of infectivity in their systems. Several of these investigators showed further that - in contrast to the situation when the diluent is physiological saline - when viral RNA is serially diluted in non-physiological media there is a direct proportionality between infectivity and the concentration of RNA (Alexander et al., 1958; Holland et al., 1960; Ellem and Colter, 1960b; Colón and Idoine, 1964).

Although most studies have indicated that 37° is the optimal temperature for productive interaction of cultured cells and viral RNA (e.g., Ellem and Colter, 1960b; Koch, 1960; Sprunt and Alexander, 1961), there is no unanimity on this point. Colón and Idoine (1964), for example, found that in the chick embryo fibroblast - EEE-RNA system, more infectious centers were formed at 25° than at 37°, and other investigators allowed RNA and cells to interact at 20° on the grounds that increased cell damage occurred at 37° with no increase in the number of plaques (Crick et al., 1966; Holland et al., 1960). Since cultured cells differ in their ability to resist osmotic shock, the assay conditions (such as time and temperature of adsorption, nature and concentration of the salt) which give optimal plaque formation would be expected to differ from one cell system to

another, and, in each, probably reflect a balance between conditions which would result in increased uptake of intact viral RNA molecules and those which cause minimal cellular damage.

Efforts have been made to minimize damage to sensitive cells by exposing them to solutions of gradually increasing salt concentration (Boeyé, 1959; Colón and Idoine, 1964). However, exposure of monolayers to the various non-physiological media necessary for efficient infection of the cells can be avoided entirely by employing a suspended cell system (Ellem and Colter, 1960a,b). In this system, cells and RNA are allowed to interact in hypertonic salt solutions, sucrose solutions, etc., after which the number of infected cells in the mixture is quantitated by titration on monolayers of "healthy" cells. Damage to cells in the monolayer is thus avoided, and well-defined plaques, uniformly distributed over the plate, are obtained.

Various theories have been advanced to explain the enhancing effect of hypertonicity on the infectivity of viral RNA preparations. It has been suggested that higher salt concentrations may maintain RNA molecules in a more compact configuration so that they are more easily taken up by cells (Sprunt and Alexander, 1961). Sprecher-Goldberger (1964) has reported that the infectivity of Sindbis virus RNA survives heating better in solutions of high ionic strength than in solutions of physiological

ionic strength, and that after heating in solutions of physiological ionic strength, more infectivity is recovered after slow than after rapid cooling. These observations prompted her to suggest that some hydrogen bonding between base pairs in the RNA is necessary for infectivity. In the case of EMC virus RNA, however, alteration of the secondary structure by reduction of the salt concentration was found to have no effect on the infectivity of the molecule (Sanders, 1964).

Several groups have examined the premise that more RNA is taken up by cells from hypertonic than from isotonic media, by measuring the amount of radiolabelled RNA that became firmly cell associated under a variety of incubation conditions. It was found that only a very small percentage of the RNA molecules exposed to cells was absorbed (Holland et al., 1960; Ellem and Colter, 1961a; Bases and Huppert, 1966). The study of Ellem and Colter (1961a), in which the uptake of radiolabelled ribosomal RNA and cell viability were both quantitated, showed that the amount of RNA taken up by viable cells was not affected significantly by changes in the tonicity of the incubation medium, while Borriess and Koch (1964) found that more RNA became attached to cells in isotonic than in hypertonic solution.

It has also been suggested that RNA is protected from degradation by ribonucleases in solutions of elevated ionic strength, since it is known that the activity of this

enzyme is inhibited under these conditions (Kalnitsky et al., 1959; Dickman and Ring, 1958). This may explain why inactivators of polio RNA were detected in isotonic but not in hypertonic washings of cell monolayers (Sprunt et al., 1961; Norman and Veomett, 1961). Colter and Ellem (1961b) suggested that sucrose solutions of elevated osmolarity may exert their stimulatory effect by dehydrating the cells, thus producing a hypertonic intracellular environment, which, in turn, might inhibit the activity of intracellular nucleases. In line with this suggestion are the observations of Smull and Ludwig (1965), who reported that dehydration of cell monolayers before exposure to viral RNA results in increased plaque formation, and that the infectivity of the RNA is not influenced by changes in the ionic strength of the inoculation medium when dehydrated monolayers are used in the assay.

Not all investigators have found that hypertonic media stimulate the infectivity of viral RNA preparations. Bachrach (1960) and Engler and Tolbert (1963) have reported that, in the FMD-RNA - bovine kidney cell and poliovirus RNA - monkey kidney cell systems respectively, hypertonic media did not increase the number of plaques produced over the number produced in buffered physiological saline. However, Engler and Tolbert did obtain an increase in the titer of polio RNA in their system by using the assay method first described by Dubes and Klingler (1960, 1961) in which the cells are depleted of Ca^{++} and insoluble

"facilitator" is used. In this method, cell monolayers are washed several times with a Ca^{++} -free medium prior to inoculation of the RNA in a solution containing a poorly water-soluble material such as $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, Cr_2O_3 , or talc. This technique, usually with minor modifications for different cell-RNA systems, has been found to be applicable to the assay of RNA's isolated from a number of different viruses (Chapin and Dubes, 1962, 1963, 1964; Faas et al., 1963; Rouhandeh, 1963, 1964). A wide variety of facilitators, differing in effectiveness, have been described. In general, the effectiveness of these materials has been found to be decreased by increasing the tonicity of the medium to hypertonic levels (Dubes and Klingler, 1961; Dubes et al., 1964). Rouhandeh (1964) has presented evidence which suggests that the infectious RNA molecules adsorb to sites on the facilitator particles, and are thus more readily taken up by Ca^{++} -depleted cells.

The assay systems which are the most sensitive, and which have been most widely used in recent years, are those which utilize a polycation of some kind. They came into being with the discovery that the titer of infectious viral RNA preparations was increased by the addition of histones (Smull et al., 1961; Smull and Ludwig, 1962). Evidence has been presented to support the suggestion that the formation of a histone-RNA complex may be involved in the enhancement of the biological activity of viral RNA. For example,

infectivity was shown to be enhanced if the RNA and histone were mixed prior to inoculation onto cell monolayers, whereas little or no infectivity was demonstrable when RNA and histone were added at different times (Smull and Ludwig, 1962). That formation of a histone-RNA complex may be dependent upon the structural configuration of the RNA molecule was suggested by the observations that the infectivity of the RNA-histone mixture is dependent upon the presence of a low concentration of a monovalent salt but is decreased in hypertonic solutions, and that divalent cations also decrease the infectivity of the mixture, - possibly by binding to sites on the RNA molecule to which histone would ordinarily attach (Ludwig and Smull, 1963).

Smull and Ludwig (1962) examined the effects of a number of other substances on the infectivity of poliovirus RNA for HeLa cell monolayers. Protamine sulfate was found to have an enhancing effect, a finding later verified by other workers (including Amstey and Parkman (1966) who reported that this material stimulated the infectivity of poliovirus RNA to a greater extent than did histones) although some Japanese investigators reported Japanese B encephalitis virus RNA to be precipitated by protamine sulfate and its infectivity for chick embryo fibroblast cells to be thereby inhibited (Igarashi, Kitano and Fukai, 1963; Igarashi et al., 1963). The enhancing effect of protamine sulfate may reflect an

increased uptake of viral RNA, since it has been shown that the uptake of labelled RNA by cells is stimulated by this basic protein (Amos and Kearns, 1963; Borriss, 1965). The non-basic proteins, bovine serum albumin and globulin, were found, in agreement with the earlier results of Alexander et al. (1958), to have no effect on the infectivity of polio RNA, and a number of small, basic molecules (spermine, spermidine, cadaverine and arginine) were also shown to have no enhancing effect. It should be noted, however, that Moscarello (1965) subsequently reported that the infectivity of EMC-RNA for L cells was increased more than ten-fold by the addition of putrescine or cadaverine to the assay system, and, on the basis of altered melting curves and the protection of RNA against degradation by low concentrations of RNase, suggested that putrescine may bind to RNA.

Rouhandeh (1965) has described the results of an interesting study in which the RNA's isolated from Cocksackie A7, ECHO 8, and three strains of poliovirus were assayed for infectivity on monolayers of HeLa and MK cells, and in which the four solutions, - 1M NaCl, 2M MgSO₄, physiological saline containing 0.5% CaHPO₄·2H₂O, and physiological saline containing 400 µg histones/ml, - were compared as diluents for the various RNA preparations. With both cell types, the hypertonic solutions were found to be much more efficient than the other two diluents with respect to stimulating the demonstrable infectivity of all the RNA preparations.

Subsequent to the description of the use of histones in the assay of viral RNA's, other polycations have been examined, and some of them have been found to be much more effective than histones in stimulating the infectivity of viral RNA preparations. These include DEAE-dextran (Vaheri and Pagano, 1965; Pagano and Vaheri, 1965; Amstey and Parkman, 1966; Bachrach, 1966; Koch et al., 1966; Dianzini et al., 1967; Koch and Bishop, 1968); poly-L-ornithine (Koch et al., 1966, Dianzini et al., 1967, Koch and Bishop, 1968); poly-L-lysine, methylated albumin, arginine-rich histone, avidin, and lysozyme (Koch and Bishop, 1968). Data presented in some of these reports suggest that the formation of a polycation-RNA complex may be an essential feature of the infectious process.

Despite the fact that assay systems employing polycations have been shown to be highly sensitive for the assay of viral RNA's in a variety of cell systems, it cannot be assumed that they are always the ones of choice. Amstey and Parkman (1966), for example, have reported that, in the poliovirus RNA-primary African green monkey kidney cell system, higher titers were obtained when cells were exposed to the RNA in a diluent containing dimethylsulfoxide (DMSO) than in a diluent containing either protamine or DEAE-dextran. DMSO is a fascinating chemical. Clinically, it has been used to stimulate the absorption (through the skin) of a wide variety of compounds (see

review by Kligman, 1965), it has been shown to disrupt the secondary structure of polynucleotides (Katz and Penman, 1966), and it has been reported that in its presence it is possible to grow and preserve mammalian cells in a simple, chemically-defined medium in the absence of serum or serum products (Brown and Nagle, 1965).

Certain other techniques, alleged to increase the efficiency of viral RNA assays, have been described. For example, Sprunt et al. (1967) incorporated bentonite in all media as an RNase inhibitor (Fraenkel-Conrat et al., 1961), although Crick et al. (1966) reported that bentonite inhibited plaque formation in their system. Koch et al. (1967) reported an enhanced formation of infectious centers in the presence of actinomycin D. Quantitatively, the stimulation they observed was not great, but it is of some interest because the action of this drug is probably intracellular, and, since the effect was reported to be different for double- and single-stranded RNA, it might be related to early events in the initiation of a virus growth cycle. Finally, the isolation of infectious RNA from suspensions of purified virus particles rather than from infected tissues eliminates, or at least reduces, the possibility that the infectivity may be inhibited by protein or heterologous nucleic acid molecules. That the presence of high molecular weight DNA in crude preparations can inhibit the biological activity of RNA has been shown by Franklin et al.

(1959), Sokol et al. (1961), and Holland et al. (1960). Engler and Tolbert (1963) have reported that a cell homogenate, if present in sufficient quantity, may inhibit the infectivity of viral RNA,- a not very surprising observation in view of the wide distribution of nucleases in cells. These investigators also found that several proteins, as well as RNA and DNA from commercial sources, inhibited the biological activity of polio RNA. A similar observation had been reported earlier by Brown et al. (1960), who also demonstrated that RNA was extensively degraded when incubated with bovine plasma albumin,- no doubt due to contamination of the protein by traces of a ribonuclease. Dubes and Rouhandeh (1963) measured the interference between infectious RNA's isolated from two antigenically-distinct types of poliovirus, and presented experimental evidence which suggested that the interference was due to competition between the two species of RNA for sites on the facilitator particles used in their assay system. Amos and Kearns (1963) have shown that radiolabelled RNA and DNA molecules compete for uptake by cells.

If the efficiency with which viruses infect susceptible cells is due to a specific interaction between cellular receptors and groups on the protein coat of the virus particle, the low levels of infectivity of nucleic acids can be explained by the lack of a specific mechanism for

attachment to and penetration of host cells. The concept that the cell-virus interaction is sufficiently specific to play a central role in determining the host range of a virus has been reinforced by the demonstration that it is possible to infect cells which are normally insusceptible to a particular intact virus with RNA isolated from that virus (De Somer et al., 1959; Mountain and Alexander, 1959; Holland et al., 1959). In such cases, the progeny resulting from the infection appear to be identical to those from which the RNA was isolated, so that only a single cycle of replication can take place. The efficiency of infection of a non-susceptible host cell with RNA is not appreciably less, and may, in some cases, be greater than that of susceptible cells (Holland and Hoyer, 1962; Schaffer, 1962). Polio RNA has been shown capable of initiating an infection leading to the production of progeny virus particles in cells of all warm-blooded animals tested. However, there do appear to be limits to the host range of viral nucleic acids. No evidence of virus production could be found following exposure of fish, frog, and plant cells, protozoa, and bacterial protoplasts to polio RNA, and production of TMV could not be detected in HeLa cells exposed to infectious TMV RNA (Holland and Hoyer, 1962). More recently, Ben Gurion and Ginzberg-Tietz (1965) claim to have successfully infected E. coli protoplasts with the RNA of EMC virus.

Since a survey of the literature suggests that "the only generalization to be made regarding tissue culture assay of infectious RNA is that optimum sensitivity and reproducibility are functions of the RNA-cell system and the conditions in the individual investigator's laboratory" (Schaffer, 1962), the first objective of the present study was to define carefully the optimal conditions for the infection of L cells by Mengo RNA. A study of the interaction of Mengo RNA with two cell lines which are insusceptible to infection with intact Mengo virus was then undertaken, in order to obtain evidence concerning the importance of the cell type in determining the conditions that are optimal for the assay of a particular infectious viral RNA. Finally, an investigation, designed to define the mechanisms by which hypertonic salt solutions, solutions of elevated osmolarity, and, more particularly, the additives DMSO and DEAE-dextran stimulate the productive interaction between cultured mammalian cells and infectious viral RNA, was carried out. The results, which comprise the main body of this thesis, are summarized in the three chapters which follow.

ROUTINE MATERIALS AND METHODS

Cells

Earle's L-929 strain of mouse fibroblasts was obtained from the American Type Culture Collection, Rockville, Md. These cells were used in studies of the interaction of cultured cells with viral RNA, for the propagation of Mengo virus, and for the titration (by the plaque method) of pools of Mengo virus.

Tissue Culture Media

All media were made up in distilled, deionized water.

Growth medium. "Basal medium, Eagle, Diploid" was obtained in powder form from the Grand Island Biological Co., Grand Island, N.Y. The powder was dissolved in deionized water and the solution sterilized by filtration. This basal medium, containing Earle's salts (Earle, 1943) and Eagle's nutrients (Eagle, 1959), was then supplemented by addition of the following (the final concentration of each is indicated):

1. Sodium bicarbonate, 0.26%
2. Horse serum (Baltimore Biological Laboratory, Baltimore, Md.), 10%
3. Aureomycin (Lederle Laboratories, Pearl River, N.Y.), 50 $\mu\text{g/ml}$; penicillin G (Ayerst Laboratories, Montreal, P.Q.), 100 I.U./ml; and streptomycin sulfate (Glaxo-Allenburys Ltd., Weston, Ont.), 100 $\mu\text{g/ml}$

Spinner medium. Identical to growth medium except that the basic powdered medium used lacked calcium - "minimum essential medium (Eagle) with spinner salts" (Grand Island Biological Co.).

Maintenance of Cells

L cells (approx. 10×10^6) in 75 ml growth medium were added to 1-liter Blake bottles (Kimble Products, Owens-Illinois Co., Toledo, Ohio) and incubated at 37° for 3 to 4 days. The cell monolayers which formed (approx. 6×10^7 cells/bottle) were detached from the glass by replacing the growth medium with 10 ml 0.25% trypsin in Hanks' balanced salt solution (Hanks and Wallace, 1949) and incubating at room temperature for 10 minutes. Cells were then collected by centrifugation, resuspended in spinner medium, and transferred to 1-liter spinner flasks (Bellco Biological Glassware, Vineland, N.J.) at a concentration of 2×10^5 cells/milliliter. The cells were kept in suspension by means of a magnetic stirring device and incubated at 37° to allow further growth to take place.

Preparation of Cell Monolayers

Cells were collected by centrifugation from spinner culture, and resuspended in growth medium to give a suspension containing 2.5×10^6 cells/6 milliliters. Six ml aliquots of this suspension were introduced into 60 mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.). Confluent monolayers containing about 6×10^6 cells were

formed after incubation for 24 hours at 37° in a humidified atmosphere of 5% CO₂ in air.

Overlay diluent. Calf serum, inactivated by heating at 56° for 45 min, was added, to a final concentration of 30%, to a solution containing three times the usual concentration of Hanks' salts, six times the usual concentration of Eagle's nutrients, 0.75% sodium bicarbonate and 0.02% phenol red.

Agar overlay. Two volumes of 1.5% Noble agar (Difco Laboratories, Detroit, Mich.) were mixed at 45° with one volume of overlay diluent.

Virus

Mengo encephalomyelitis virus was obtained originally by Dr. J.S. Colter from Dr. K. Smithburn of the Division of Medicine and Health of the Rockefeller Foundation. Subsequently, three variants were isolated by Ellem and Colter (1961b) from pools prepared by propagating the virus in Ehrlich ascites carcinoma cells in vivo. The M-Mengo variant, which produces sharply defined, medium-sized plaques in monolayers of L-929 cells under agar overlay, was used exclusively in the studies reported in this thesis.

Growth of Virus

Approximately 50×10^6 L-929 cells in 100 ml of growth medium (containing 5% horse serum) were added to 2-liter

Povitsky bottles and incubated at 37° until a confluent monolayer of approximately 100×10^6 cells had formed (usually 24 hours). The medium was discarded and 20 ml of a virus suspension were added. The multiplicity of infection was 5 to 10 pfu per cell. After a 1-hour incubation at 37°, 100 ml of growth medium (containing 1% horse serum) were introduced. Incubation was continued for 18 to 24 hours, at which time the cell mass could be dislodged from the glass by gentle shaking. Lysates from 8 to 12 bottles were pooled and centrifuged at 850 g for 10 minutes. The pellet was resuspended in a few ml of the supernatant fluid, frozen and thawed several times to release attached virus, and centrifuged again. The two supernatants were combined, and cooled in an ice bath. Methanol was added to a final concentration of 35% (v/v), and the solution was allowed to stand for at least 4 hours at 4°. The precipitated material was collected by sedimentation at 8000 g, resuspended in 16 ml of Tris-HCl buffer (pH 8.0), frozen and stored at -20°.

In the later stages of this work, the number of virus-producing cells per unit volume of culture medium was increased by using roller bottles. The large cylindrical bottles (Bellco Biological Glassware, Vineland, N.J.) were first coated with fetal calf serum (Baltimore Biological Laboratory, Baltimore, Md.) to facilitate attachment of cells, after which 150 ml of growth medium (containing 5%

horse serum) and L cells to give a concentration of about 10^6 cells/ml were added. The bottles were rotated on a Bellco roller apparatus in a 37° room until confluent monolayers of approximately $5-6 \times 10^8$ cells per bottle had formed (48 hours). The growth medium was removed from the cell monolayers, and replaced with 50 ml of a suspension of Mengo virus at a concentration of approximately 5×10^7 pfu per milliliter. After rotation of the bottles for 2 hours to permit the virus to attach, 100 ml of growth medium (1% horse serum) were added to each bottle and incubation was continued for 24 hours. At the end of this time the cell mass could be dislodged by shaking. The cell lysates were treated in the same manner as were those prepared in Povitsky bottles.

Isolation of RNA

One or two of the methanol-precipitated virus preparations were thawed, and PBS was added to bring the volume to 50 milliliters. The suspension was then made 0.02 M and 0.25% with respect to EDTA and sodium deoxycholate respectively and placed in an omnimixer cup in an ice bath. An equal volume of water-saturated phenol was added, and the contents of the cup were mixed for 10 minutes. The resulting emulsion was centrifuged at 2000 g for 10 min, after which the upper aqueous layer was removed, and shaken for 5 min with an equal volume of water-saturated phenol. After a 10 min centrifugation at 2000 g, the upper

Table M.1

Recovery of L cells after washing in various media

Wash Medium	Cells/ml	% Viable Cells
Growth medium	377,000	98
Growth medium minus serum	192,000	96
PBS	122,000	94
PBS containing 10% horse serum	373,000	98
PBS containing 0.25% BPA	395,000	99

Cells were collected by centrifugation from 10 ml aliquots of a spinner culture, washed 3x in 5 ml volumes of the media listed in the table, and were then resuspended in 10 ml volumes of growth medium. Cell counts were made using a haemocytometer, and cell viabilities were determined by neutral red uptake.

Cell concentration in spinner culture = 386,000/ml (99% viable).

Table M.2

The effect of washing cells on infectious center formation in L cell - Mengo RNA mixtures

Number of Washes	Infectious centers/ 10^6 cells/ml RNA	
	Sucrose	Sucrose/DMSO
0	2,600	2,800
1	6,900	19,600
2	6,800	72,000
3	9,000	140,000

L cells were collected by centrifugation from aliquots of a spinner culture, washed in PBS containing 0.05% BPA, and then incubated for 5 min at 37° with Mengo RNA dissolved in either 0.6 M Sucrose/PBS or 0.6 M Sucrose/PBS containing 10% DMSO.

layer was carefully removed to avoid disturbing the interface, and residual phenol was removed therefrom by five successive extractions with ether. Finally, nitrogen gas was bubbled through the RNA solution until ether was no longer detectable, and the solution was frozen in aliquots and stored at -60° .

Assay of Infectious RNA

Cells used in studies of cell-RNA interaction. It was found, in preliminary experiments, that washing L cells with either the phosphate-buffered physiological saline (PBS) of Dulbecco and Vogt (1954) or with other protein-free media, resulted in a substantial loss of cells. However, the addition of serum or bovine plasma albumin (BPA) (fraction 5, Pentex Inc., Kankake, Ill.) to PBS prevented this unexpected loss. This finding is illustrated by the data shown in Table M.1. Additional studies established that the minimal concentration of BPA (in PBS) required to prevent cell loss during repeated washings was 0.05%. In all the studies described herein, PBS containing 0.05% BPA was used as the wash solution.

The number of times that L cells were washed was found to have a profound effect on the number of infectious centers formed when the cells were subsequently incubated with Mengo RNA. From the data summarized in Table M.2 it may be seen that the number of infectious centers produced

in a fixed number of L cells was found to increase progressively as the number of washes was increased from one to three,- probably due to the removal of nucleases associated with the cells. Since additional washes did not increase significantly the number of infectious centers formed, the routine procedure adopted was to wash cells three times before using them for studies of cell-RNA interaction.

Titration of infectious RNA. Mengo RNA preparations were assayed for infectivity using the suspended cell or infectious center technique described by Ellem and Colter (1960a). In brief, the procedure followed was as outlined below.

A known number of cells (harvested from spinner culture and washed three times in PBS containing 0.05% BPA) were resuspended in a solution of RNA (usually a 1:10 or 1:20 dilution of the stock RNA solution) in the medium to be examined (prewarmed to 37°), and the suspension (usually containing 2.5×10^6 cells/ml) was placed in a water bath at 37°. All incubation media contained EDTA at a final concentration of 0.01M (Ellem and Colter, 1961a). At intervals, aliquots of the suspension were removed and diluted 10-fold into growth medium. Further dilutions of the suspensions were usually made in order to reduce the concentration of infectious centers and thus to keep the number of plaques within countable limits. Aliquots of

these suspensions were titrated for infectious centers on preformed monolayers of L cells. This involved removing the medium from monolayers (grown in 60 mm plastic petri dishes), and replacing it with 4 ml aliquots of the suspensions of treated cells. The petri dishes were then incubated at 37° in a humidified atmosphere of 5% CO₂ in air for 90 min to allow the suspended cells to settle out and become attached to the monolayer. The medium was then removed and 4.5 ml of agar overlay was applied to each plate. After a further two-day incubation at 37°, 3 ml of agar overlay containing neutral red at a concentration of 1:10,000 (w/v) were added to each plate. Virus plaques were scored when visible several hours later. Additional plaques were counted 24 hours later, by which time plaque counts reached maximum levels.

It should be noted that dilution of the cell-RNA mixtures into growth medium effectively blocks any further cell-RNA interaction, and makes it possible to define, with reasonable precision, the kinetics of the interaction (Ellem and Colter, 1960a,b).

Sterilization Procedures

The basic media, neutral red solutions, and balanced salt solutions were sterilized by filtration through a Seitz filter, the first portion of each filtrate being discarded. Sucrose solutions were sterilized by free-flowing steam in an autoclave for 15 minutes. Agar

suspensions were autoclaved at 125° for 15 minutes. All solutions were tested for sterility by incubating samples in sterile thioglycollate medium (Baltimore Biological Laboratory, Baltimore, Md.) and sterile brain heart infusion medium (Difco Laboratories, Detroit, Mich.).

Glassware which had been in contact with virus was soaked overnight in Wescodyne (West Chemical Products, Montreal, P.Q.), rinsed, washed thoroughly with Wedac detergent (West Chemical Products), rinsed again, and sterilized by heating in an oven at 215° for 2.5 hours. Pipets were immersed in Wescodyne for 24 hours, rinsed, immersed in concentrated H_2SO_4 for an additional 24 hours, rinsed again, and sterilized in an oven at 215° for 2.5 hours. The final rinsing of all glassware was done with deionized water.

CHAPTER I

The Interaction of Mengo RNA with L Cells: Effects of DMSO, DEAE-D, Putrescine and Polyornithine

Introduction

The initial objective of this study was to determine the optimal conditions for infection of cultured L-929 mouse fibroblast cells by RNA isolated from Mengo encephalomyelitis virus. It was not considered either feasible or profitable to examine all the methods which have been used to increase the sensitivity of assays of infectious viral RNA, and for this reason the study was limited to an examination of those methods which appeared most likely to give the greatest efficiency of infection. This chapter summarizes the data obtained from a careful examination of the effects of several additives on the interaction between Mengo RNA and L cells, and describes the optimal conditions for interaction in the presence of those compounds which were found to stimulate infectious center formation in this system.

Materials and Methods

RNA Assay

The infectious center or suspended cell method described in the Routine Materials and Methods section was used for all the studies summarized in this chapter, except where otherwise noted. A suspended, single-cell

system is preferable to a monolayer for quantitative studies of cell-RNA interaction, since the cell population can be uniformly exposed to modifications in the environment in a precisely quantitated fashion, and since, as noted previously, it permits the investigator to define the kinetics of the interaction with some precision.

All data pertaining to the infectivity of RNA preparations shown in the figures in this chapter are expressed as infectious centers formed/ 10^6 cells/milliliter of undiluted RNA solution.

A method of RNA assay, in which suspended cells (rather than monolayers) were used for plaque development as well as for infectious center formation was also used in certain of the studies described herein. The procedure, basically the same as that described by Koch et al. (1966), was as follows: L cells were washed once in PBS containing 0.05% BPA, and resuspended in PBS (containing 0.01M EDTA) at a concentration of 1.5×10^6 cells/milliliter. The cells were added, in 0.6 ml aliquots, to tubes containing 0.1 ml volumes of a solution of RNA (made in PBS containing 100 μ g DEAE-D/ml). After incubation at 37° for 5 minutes, 0.1 ml of a polyornithine (mol. wt. 21,000) solution (100 μ g/ml in PBS) was added to each cell-RNA mixture, and incubation at 37° was continued for another 60 minutes. After the

60 minute incubation period, 0.7 ml of a suspension of "indicator cells" (1.5×10^7 cells/ml in growth medium, with or without serum) was added to each tube. After mixing, the contents of each tube were added to a second tube containing 1 ml of melted (56°) agar medium (prepared by mixing one volume of 4.2% agar and two volumes of growth medium containing 2% serum). The cell-agar suspensions were then poured quickly into 60 mm plastic petri dishes containing 5 ml of solidified agar overlay as a basal layer. After incubation for 48 hrs at 37° in a humidified atmosphere of 5% CO_2 in air, the plates were stained with 3 ml of agar overlay containing neutral red (final concentration 1:10,000), and the plaques were counted when they became visible.

DEAE-dextran, prepared from dextran of molecular weight approximately 2×10^6 was purchased from Pharmacia, Uppsala, Sweden. Stock solutions containing 10 mg/ml were made up in PBS and stored at -20° .

Dimethylsulfoxide (DMSO) was purchased from Eastman Organic Chemicals and putrescine from Calbiochem.

Poly-L-ornithine, molecular weight 150,000, was obtained from Pilot Chemicals, Watertown, Mass. while the polyornithine of molecular weight 21,000 was purchased from Mann Research Laboratories, New York, N.Y.

Results

Infectious Center Formation in Solutions of Sucrose in Phosphate Buffered Physiological Saline

Ellem and Colter (1960a) carried out a study of infectious center formation in L cell-Mengo RNA mixtures incubated in buffered (0.02 M sodium phosphate, pH 7.3) hypertonic sodium chloride solutions, and in solutions containing varying concentrations of sucrose in 0.14 M NaCl - 0.02 M sodium phosphate, pH 7.3 (x M sucrose/PBS). They found that, of all the solutions in these two series, the maximum number of infectious centers was formed in the solution containing 0.7 M sucrose (i.e. 0.7 M sucrose/PBS). The present study was initiated by a re-examination of this earlier work. Infectious center formation in L cell-Mengo RNA mixtures incubated in PBS and in PBS containing sucrose at concentrations ranging from 0.1 to 0.7 M was measured. Some degree of stimulation (relative to PBS) was observed at all sucrose concentrations, but, on the basis of repeated experiments, 0.6 M sucrose/PBS was found to be the optimal incubation medium in this series. Since the differences between the numbers of infectious centers formed in 0.6 M sucrose/PBS and 0.7 M sucrose/PBS were always very small, it was considered that this preliminary study essentially verified the earlier findings of Ellem and Colter.

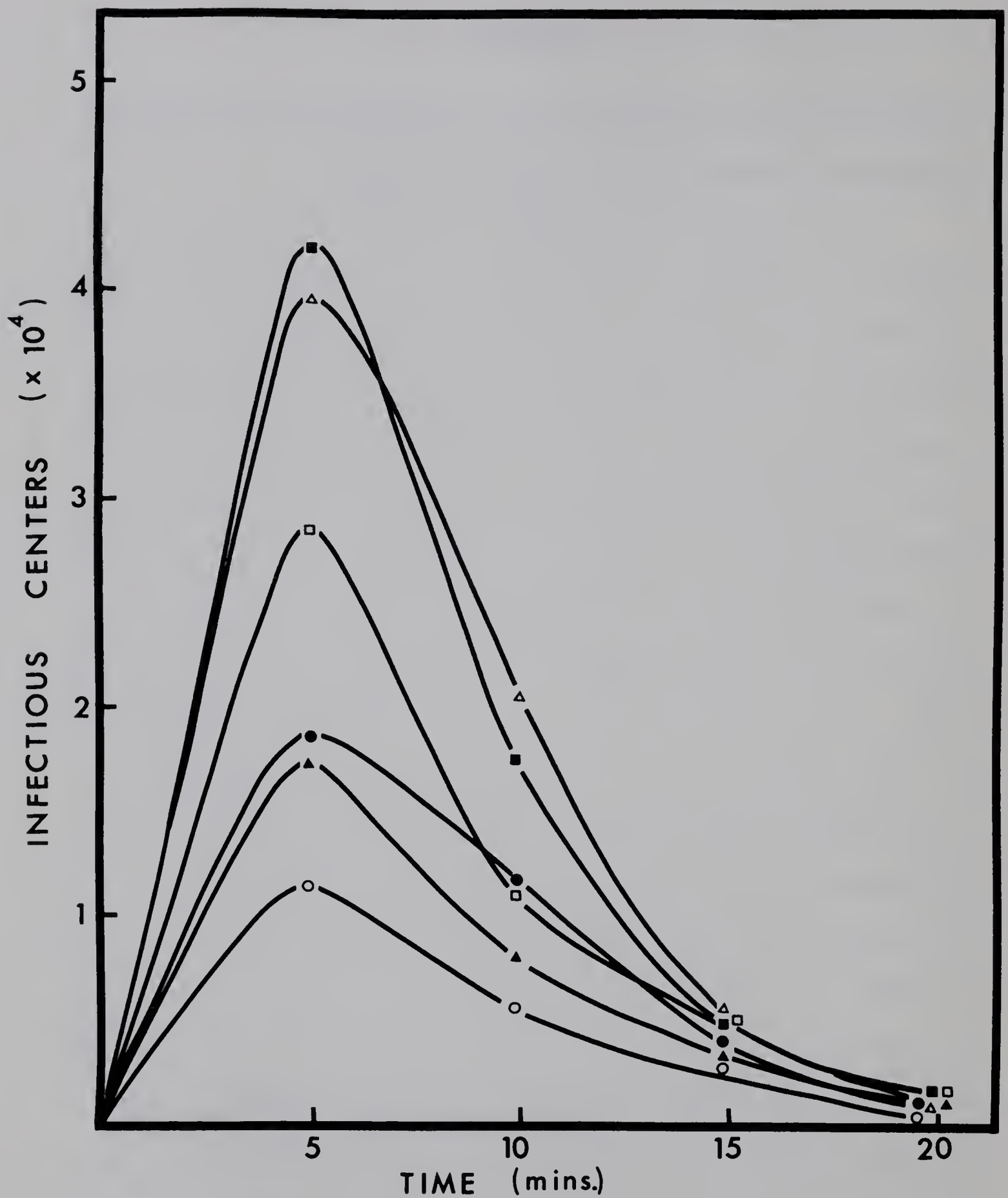
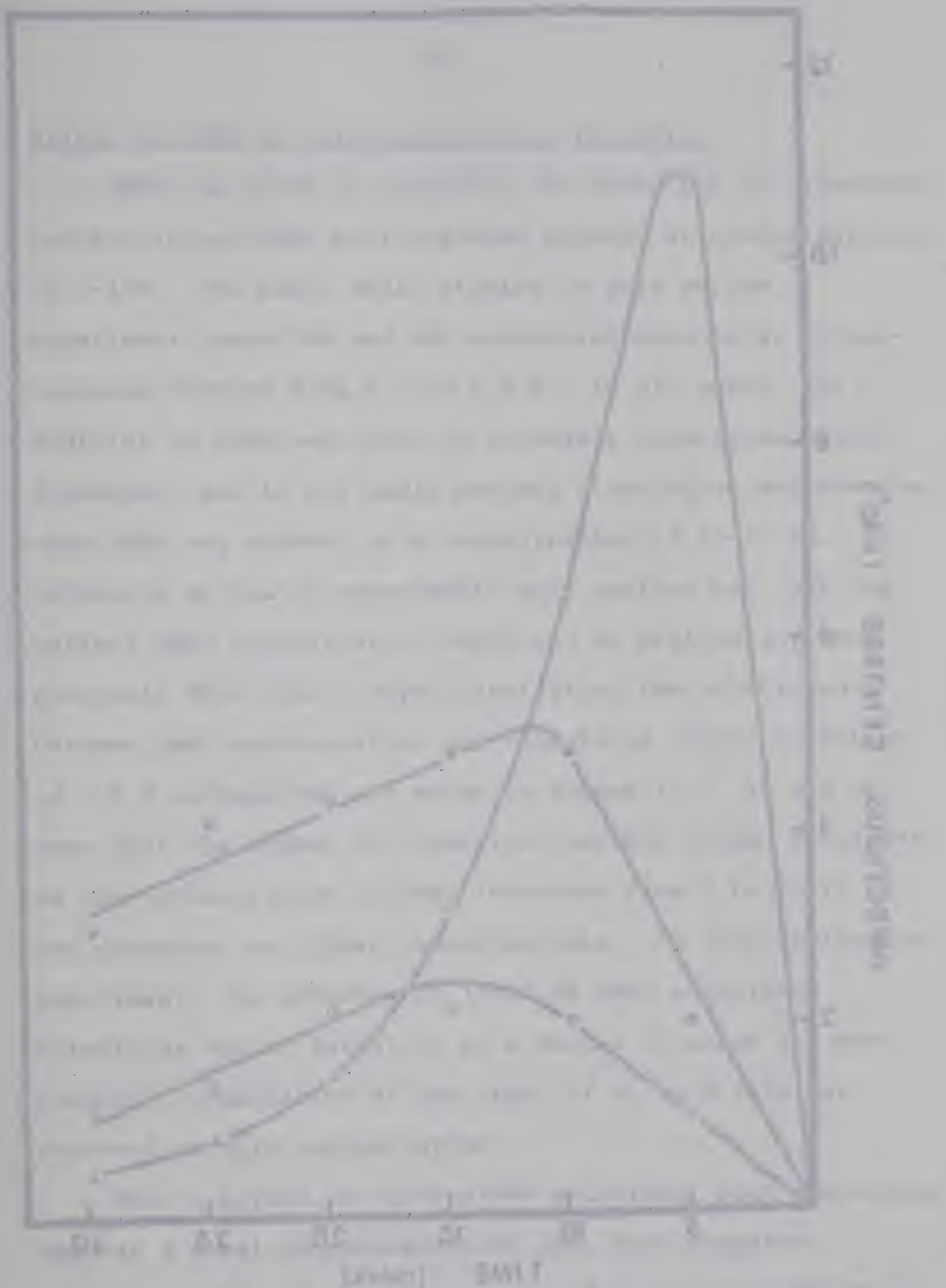


Figure 1.1. The effect of DMSO concentration on infectious center formation in L cell-Mengo RNA mixtures incubated at 37°C in 0.6 M sucrose/PBS. (○) 0% DMSO, (▲) 5% DMSO, (●) 7.5% DMSO, (△) 10% DMSO, (■) 12.5% DMSO, (□) 15% DMSO.



Intensity is a measure of the power of light per unit area. It is a scalar quantity. The unit of intensity is W m^{-2} . The intensity of light is directly proportional to the square of the amplitude of the light wave. The intensity of light is inversely proportional to the square of the distance from the source. The intensity of light is a measure of the energy carried by the light wave per unit area per unit time.

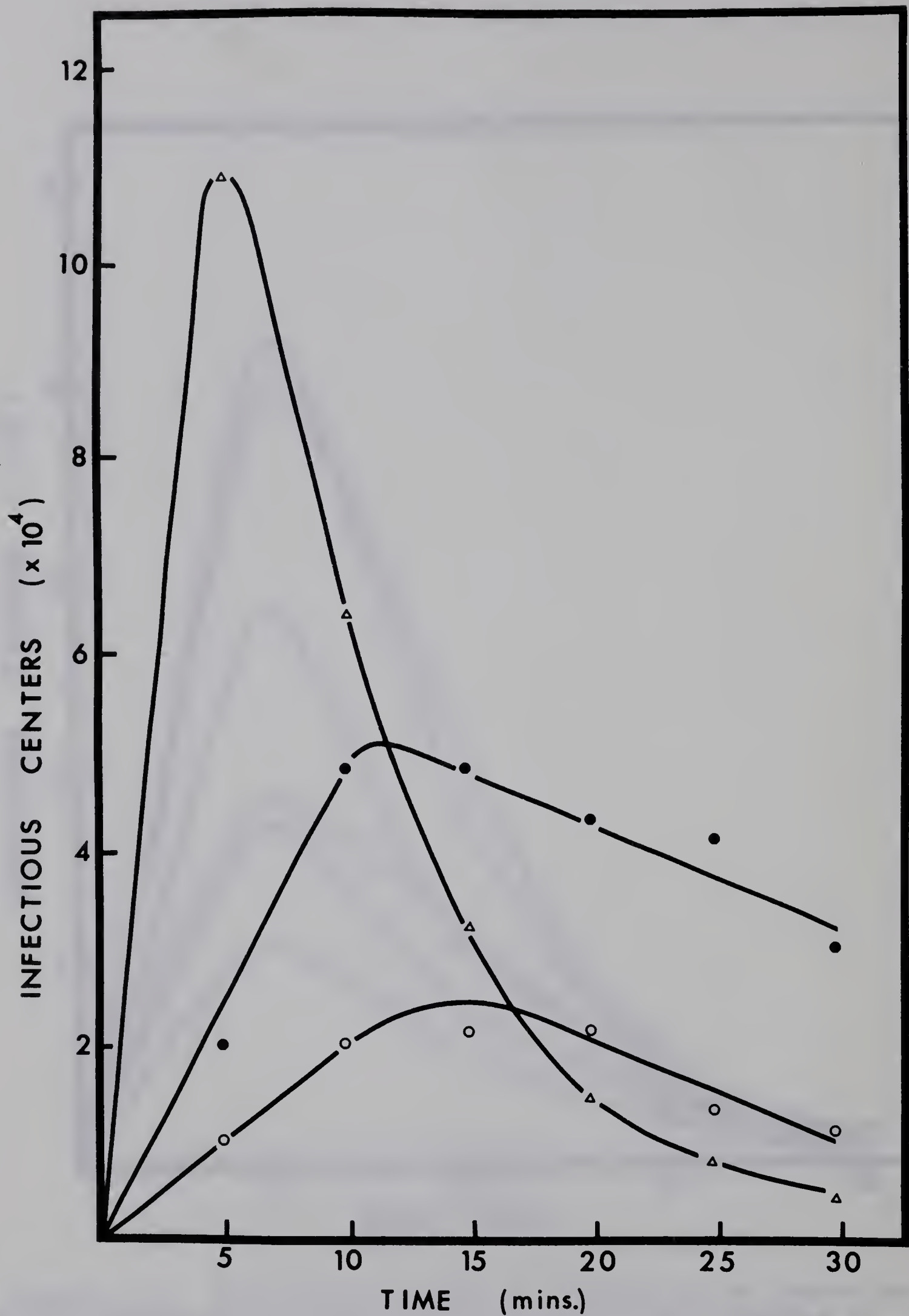


Figure 1.2. Infectious center formation in L cell-Mengo RNA mixtures incubated at 37°C in (o) PBS, (●) 0.3 M sucrose/PBS, (Δ) 0.6 M sucrose/PBS. All media contained 10% DMSO.

Effect of DMSO on Infectious Center Formation

DMSO was found to stimulate the formation of infectious centers in cell-RNA mixtures when present at concentrations of 5-15%. The basic media studied in this series of experiments were PBS and PBS containing sucrose at concentrations ranging from 0.1 to 0.6 M. In all media, the addition of DMSO was found to stimulate infectious center formation, and in all media maximal stimulation was observed when DMSO was present at a concentration of 10-12.5%. An extensive series of experiments were carried out, but the optimal DMSO concentration could not be defined any more precisely than this. Data illustrating the relationship between DMSO concentration and infectious center formation in 0.6 M sucrose/PBS are shown in Figure 1.1. It may be seen that the number of infectious centers formed increases as the concentration of DMSO increases from 0 to 10-12.5% and decreases at higher concentrations. In this particular experiment, the presence of 10-12.5% DMSO stimulated infectious center formation by a factor of about 4. More commonly, stimulation of the order of 6- to 8-fold was observed at this concentration.

When a series of sucrose/PBS solutions, each containing DMSO at a final concentration of 10%, were compared directly, maximal infectious center formation was found in 0.6 M sucrose/PBS containing DMSO. This is clear from Figure 1.2 in which data obtained from an examination of

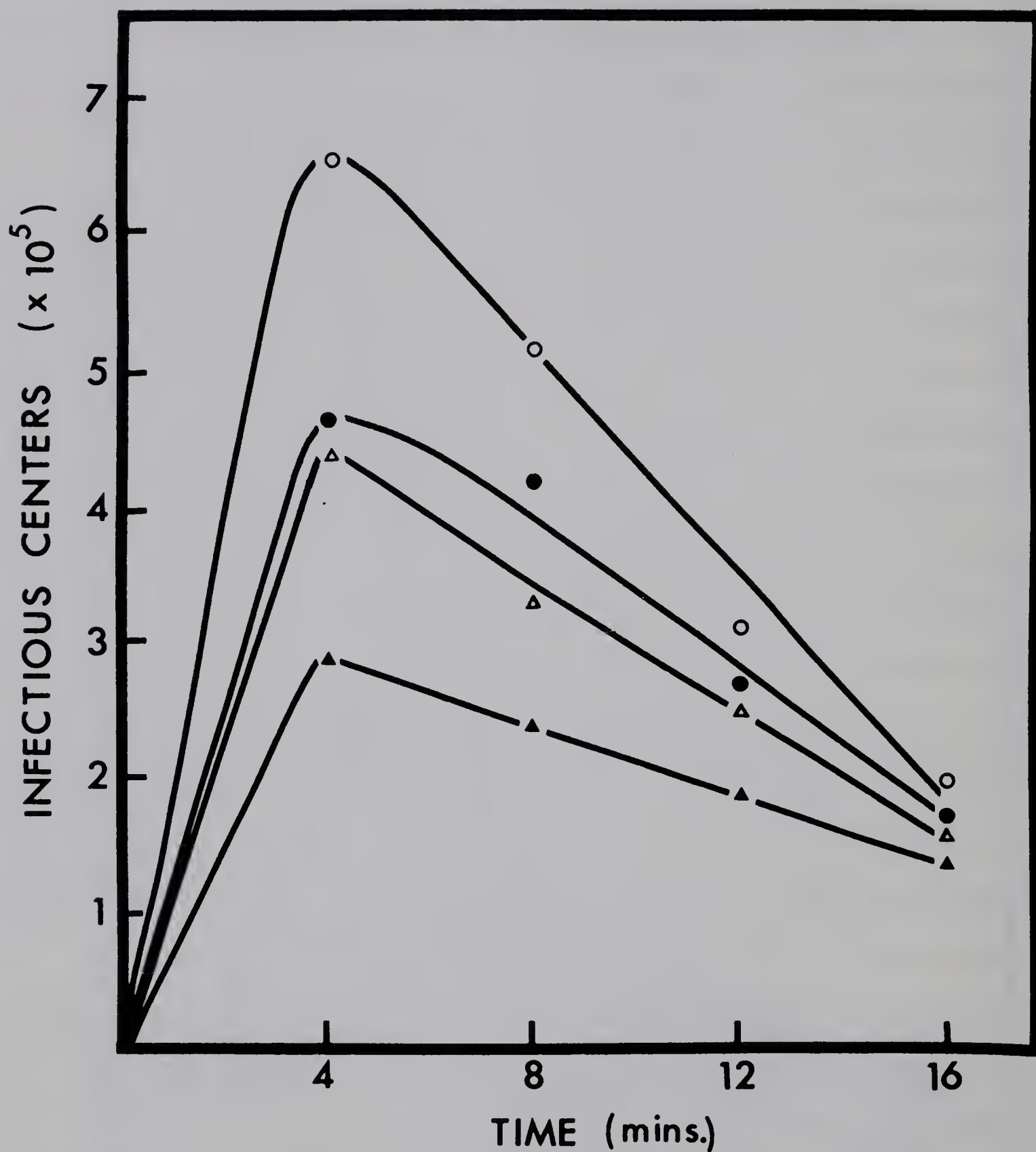


Figure 1.3. The effect of DEAE-D concentration on the formation of infectious centers in L cell-Mengo RNA mixtures incubated at 37°C in PBS. (o) 250 µg/ml, (●) 500 µg/ml, (Δ) 750 µg/ml, (▲) 1000 µg/ml.

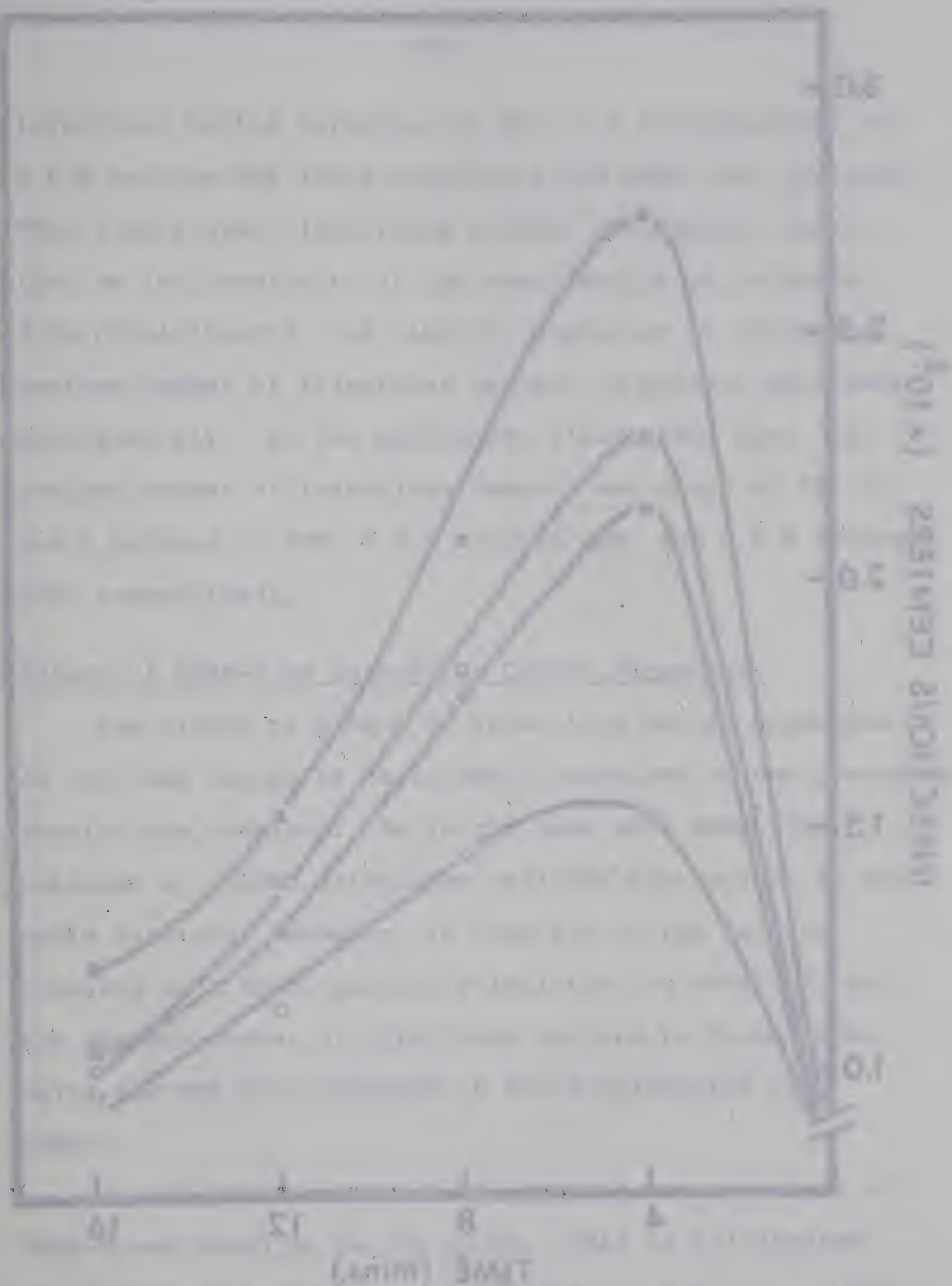


Figure 1. The effect of the concentration of the reactants on the rate of the reaction. The curves are for: (1) 0.1 M, (2) 0.2 M, (3) 0.3 M, and (4) 0.4 M. The reaction was carried out at 25°C.

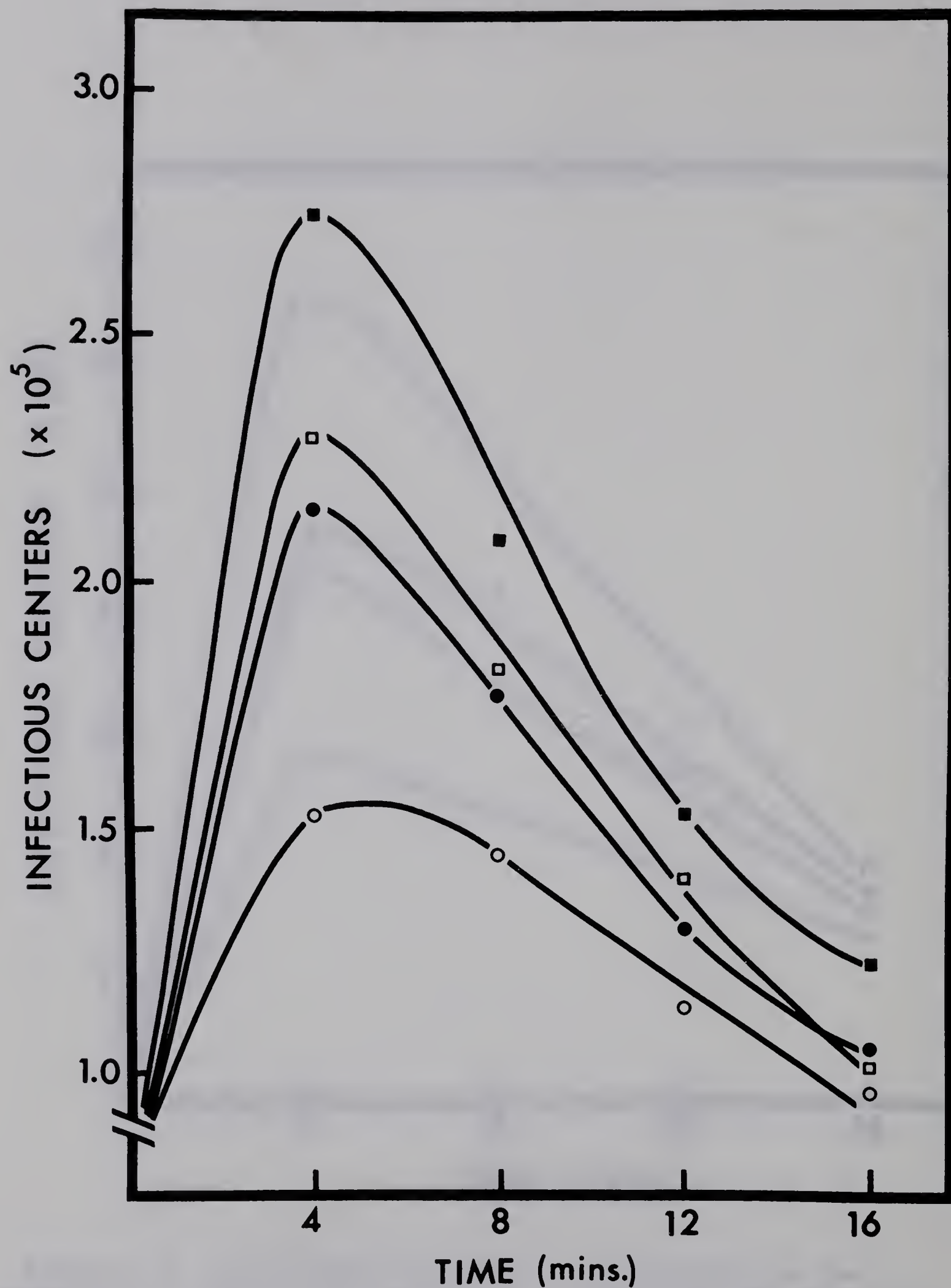


Figure 1.4. The effect of DEAE-D concentration on the formation of infectious centers in L cell-Mengo RNA mixtures incubated at 37°C in PBS. (\square) 50 $\mu\text{g/ml}$, (\blacksquare) 100 $\mu\text{g/ml}$, (\bullet) 150 $\mu\text{g/ml}$, (\circ) 250 $\mu\text{g/ml}$.

infectious center formation in PBS, 0.3 M sucrose/PBS and 0.6 M sucrose/PBS (each containing 10% DMSO) are presented. This figure also illustrates another phenomenon, namely, that as the osmolarity of the basic medium is increased from physiological, the time of incubation at which the maximum number of infectious centers is present decreases progressively. In the experiment illustrated here, the maximum number of infectious centers was found at 15, 10, and 5 minutes in PBS, 0.3 M sucrose/PBS, and 0.6 M sucrose/PBS, respectively.

Effect of DEAE-D on Infectious Center Formation

The effect of DEAE-D on infectious center formation in the same series of basic media described in the preceding section was examined. As is the case with DMSO, the presence of DEAE-D stimulates cell-RNA interaction in all media examined. However, in contrast to the results obtained with DMSO, maximum stimulation is observed, and the maximum number of infectious centers is formed when cells and RNA are incubated in PBS supplemented with DEAE-D.

In all media examined, the optimal concentration of DEAE-D was found to be 100 $\mu\text{g/ml}$. This is illustrated, for the basic medium PBS, in Figures 1.3 and 1.4, in which the effects of two ranges of DEAE-D concentration (250-1000 $\mu\text{g/ml}$ and 50-250 $\mu\text{g/ml}$) are shown. It should be pointed out that two different RNA preparations were used

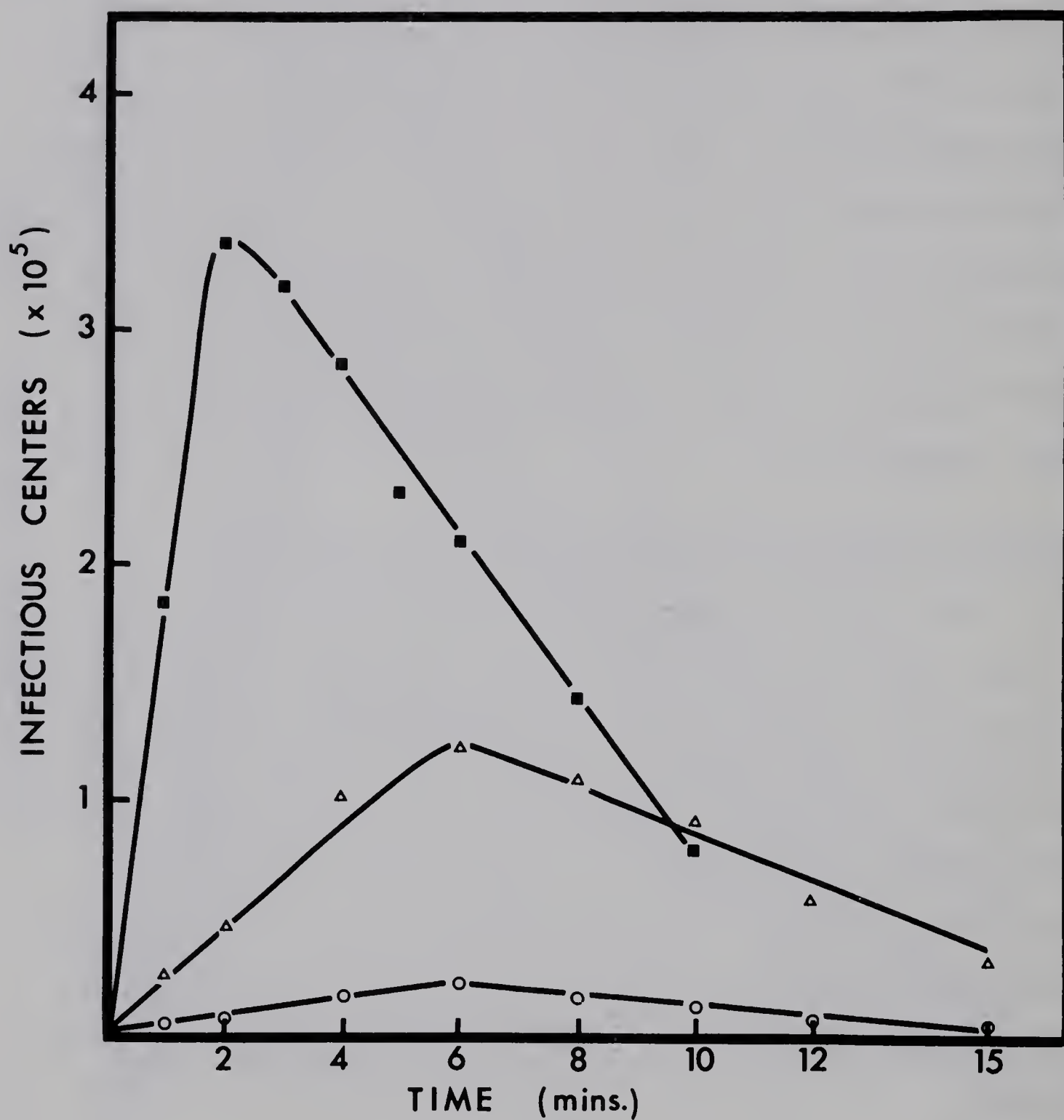


Figure 1.5. Rate of infectious center formation in L cell-Mengo RNA mixtures incubated at 37°C in (o) 0.6 M sucrose/PBS, (Δ) 0.6 M sucrose/PBS-10% DMSO, (\blacksquare) PBS containing 100 μ g DEAE-D/ml.

for the two series of experiments that are summarized in these figures. The preparation used in those experiments in which DEAE-D at concentrations ranging from 250-1000 $\mu\text{g/ml}$ (Fig. 1.3) was used was clearly of higher titer than the one used in the other series.

The data presented in Figures 1.3 and 1.4 were obtained by averaging the results of several experiments in each case. In individual experiments, maximum numbers of infectious centers were found to be produced at concentrations of DEAE-D ranging from 50 to 500 $\mu\text{g/milliliter}$.

Rate of Cell-RNA Interaction

A careful study of the rates at which infectious centers are formed in 0.6 M sucrose/PBS, 0.6 M sucrose/PBS containing 10% DMSO, and PBS containing 100 $\mu\text{g DEAE-D/ml}$ was carried out. The results are shown in Figure 1.5. In 0.6 M sucrose/PBS, with or without DMSO, the maximum number of infectious centers is present after 6 minutes of incubation at 37° . When the incubation medium is PBS containing DEAE-D, the optimal incubation time is 2 or 3 minutes. In the experiment illustrated by Figure 1.5, the optimal incubation time was found to be 2 minutes. In repeated experiments, the maximum number of infectious centers formed in this medium was found at either 2 or 3 minutes.

A rather puzzling feature of the curve relating infectious center formation to time of incubation in

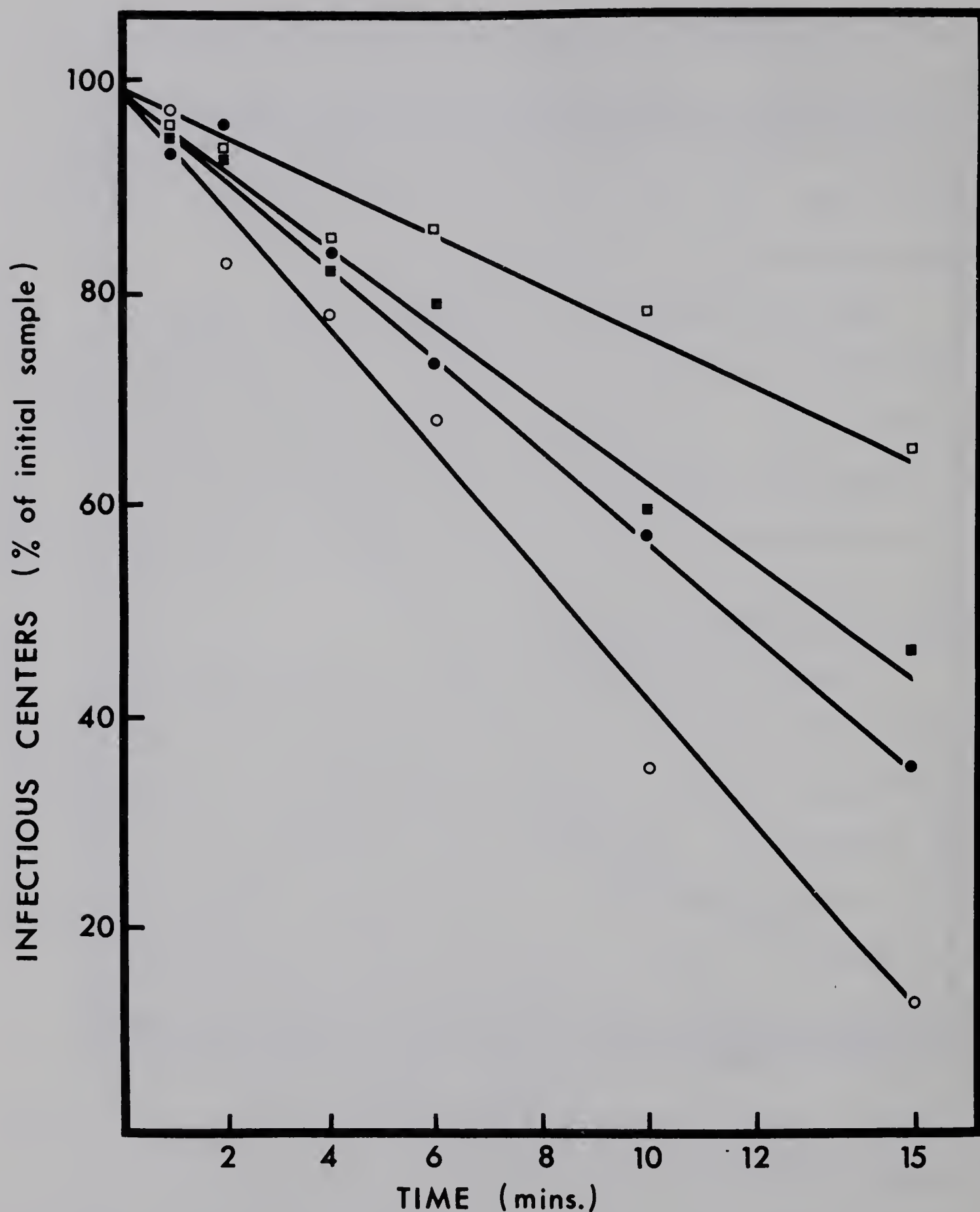


Figure 1.6. Survival of infectious centers in (o) 0.6 M sucrose/PBS, (●) 0.6 M sucrose/PBS-10% DMSO, (□) PBS and (■) PBS containing 100 µg DEAE-D/ml. Aliquots of L cells were infected with a high multiplicity of the M variant of Mengo virus, washed free of unattached virus, and suspended in the above media at 37°C. At intervals, samples were removed and assayed for infectious centers on indicator monolayers of L cells.

PBS-DEAE-D is the very rapid decrease in the number of infectious centers present when the incubation is continued beyond the optimal time of 2-3 minutes.

In an attempt to gain some insight into this phenomenon, a study of the rate at which cells lose viability in PBS, with and without DEAE-D, and in 0.6 M sucrose, with and without DMSO, was carried out. Viability was assessed by measuring the ability of the cells to support viral replication. A known number of cells was infected with a high multiplicity of Mengo virus (M variant), unattached virus was removed by washing, and the cells were resuspended in the solution to be examined. Aliquots of the suspensions were removed at intervals, diluted 1:10 in growth medium, and the number of infected cells present determined in the usual way. The results of this study are summarized in Figure 1.6.

The number of viable cells was found to decrease in a linear fashion with time of incubation in all four media. As would be expected, loss of cell viability is slowest in PBS. DMSO was found to offer a degree of protection against loss of viability to those infected cells suspended in 0.6 M sucrose/PBS. Cell viability was found to be lost more rapidly in PBS containing DEAE-D than in PBS alone. However, the rate at which cell viability is lost in this medium does not appear to be rapid enough to account for the precipitous drop in the number of infectious centers which is observed

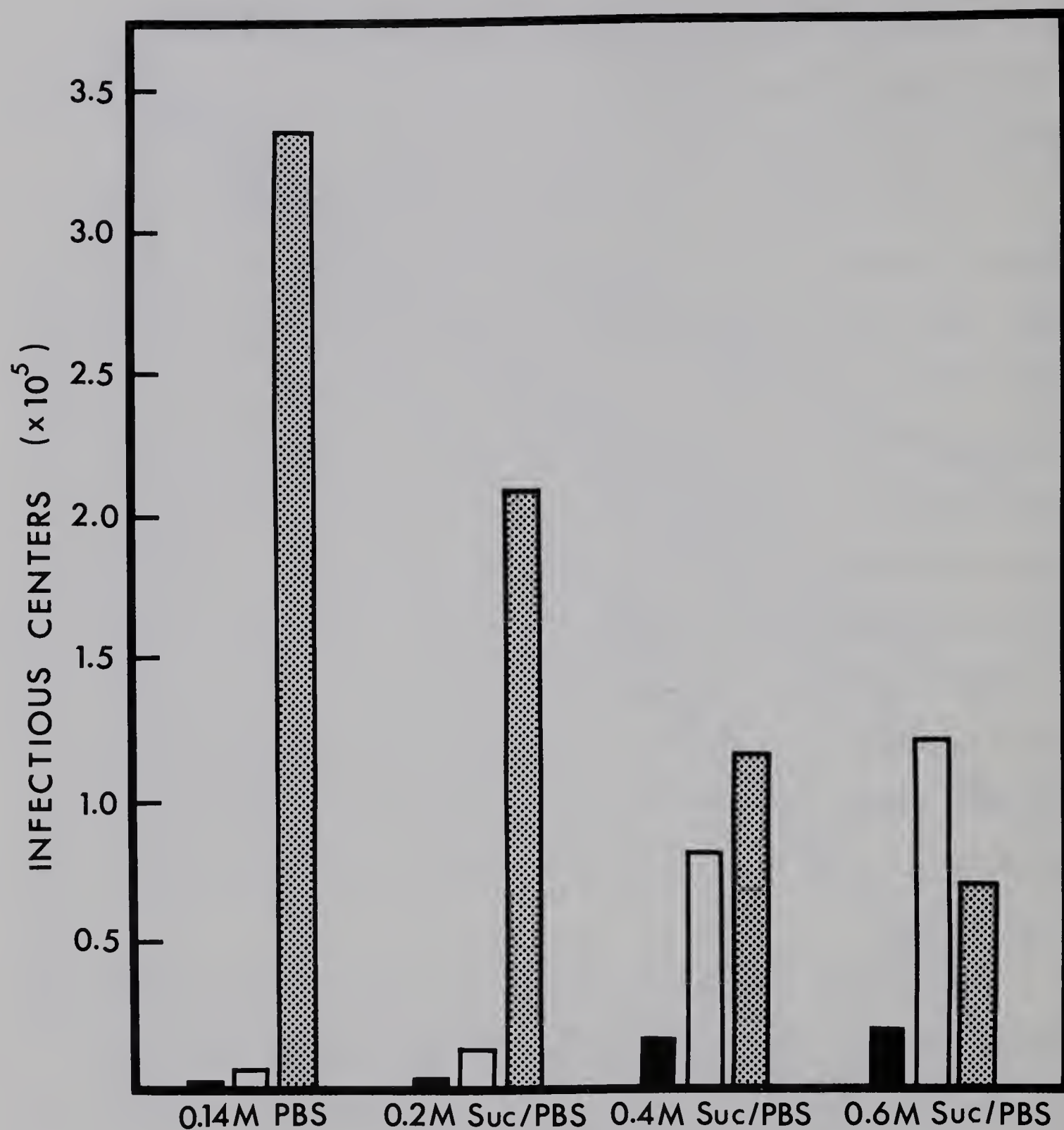


Figure 1.7. Comparative effects of DMSO and DEAE-D in stimulating infectious center formation in L cell-Mengo RNA mixtures. The basic incubation media are shown on the horizontal axis.

- Infectious center formation in the unsupplemented media.
- Infectious center formation in the basic media containing 10% DMSO.
- Infectious center formation in the basic media containing 100 μ g DEAE-D/ml.

when incubation of a cell-RNA mixture in this medium is continued for longer than 2-3 minutes.

Comparative Effects of DMSO and DEAE-D in Stimulating Cell-RNA Interaction

The addition of either DMSO or DEAE-D to any of the basic media previously described stimulates the formation of infectious centers. However, the degree of stimulation obtained depends on both the basic medium and the additive used. With unsupplemented media, the efficiency of the assay increases progressively as the sucrose concentration is increased to 0.6 M, and the addition of 10% DMSO to each stimulates infectious center formation by a factor of 4-8. With DEAE-D, on the other hand, both the degree of stimulation and the absolute number of infectious centers formed decreases progressively as the osmolarity of the basic medium is increased. These observations are illustrated by the data presented in Figure 1.7, from which it is clear that the optimal medium among those examined is PBS containing 100 μ g DEAE-D/milliliter. It should be pointed out that, in compiling the data shown in Figure 1.7, the time of incubation (at 37⁰) used with each medium was that which had been established to be optimal for infectious center formation in that medium.

The stimulatory effects of DMSO and DEAE-D are not additive. It was found that, within the limits of experimental error, the same number of infectious centers were

Table 1.1

Effect of putrescine on infectious center formation
in L cell-Mengo RNA mixtures

Incubation Medium	Infectious Centers/ 10 ⁶ cells/ml RNA
0.6 M sucrose/PBS	16,000
0.6 M sucrose/PBS containing 0.005 M putrescine	13,600
0.6 M sucrose/PBS containing 0.01 M putrescine	13,810
0.6 M sucrose/PBS containing 0.02 M putrescine	16,940
0.6 M sucrose/PBS containing 0.03 M putrescine	15,300
0.6 M sucrose/PBS containing 0.04 M putrescine	17,730
0.6 M sucrose/PBS containing 0.05 M putrescine	15,170
0.6 M sucrose/PBS containing 1.0 M putrescine	12,700

Washed L cells (2.5×10^6 cells/ml) were incubated at 37° with three different concentrations of Mengo RNA (1:10, 1:20 and 1:40 dilutions of the stock solution) in each of the media listed. The results tabulated are expressed as I.C.'s produced/10⁶ cells/ml of undiluted RNA stock solution, and are the means of values obtained from three separate experiments.

formed in cell-RNA mixtures incubated in PBS-DEAE-D (100 μ g/ml) containing 10% DMSO as in the corresponding DMSO-free medium. Similarly, the two media, 0.6 M sucrose/PBS - DEAE-D (100 μ g/ml) and 0.6 M sucrose/PBS - DEAE-D (100 μ g/ml) containing 10% DMSO, were shown to be equally efficient in promoting infectious center formation.

Effect of Putrescine on Infectious Center Formation

Moscarello (1966) reported that the number of infectious centers produced in mixtures of L cells and encephalomyocarditis virus RNA (incubated in 0.6 M sucrose/PBS) was increased 22- and 16-fold by the inclusion in the incubation mixture of 0.05 M putrescine and 0.08 M cadaverine respectively. The effects of putrescine in L cell-Mengo RNA mixtures were examined for two reasons. First, it was considered of some interest to simply compare its effect with that of DEAE-D. Second, since it seemed unlikely that putrescine and DMSO could stimulate infectious center formation by the same mechanism, the possibility that the two additives might act synergistically in our assay system was entertained. The results were surprising.

Data obtained from a study of the effect of putrescine on infectious center formation in L cell-Mengo RNA mixtures incubated in 0.6 M sucrose/PBS are summarized in Table 1.1. Infectious center formation was measured in media containing a wide range of putrescine concentrations, and, at each putrescine concentration, three different dilutions (1:10,

Table 1.2

Effect of putrescine on infectious center formation
in L cell-Mengo RNA mixtures

Incubation Medium	Infectious Centers/ 10^6 cells/ml RNA	
	4 mins	8 mins
PBS	650	800
PBS containing 100 μ g DEAE-D/ml	233,000	150,000
PBS containing 0.04 M putrescine	210	150
PBS containing 100 μ g DEAE-D/ml and 0.04 M putrescine	136,000	114,000

Mengo RNA-L cell mixtures (containing 2.5×10^6 cells/ml) were incubated at 37° in the media listed. After 4 and 8 mins of incubation, 1 ml aliquots were removed, and titrated for infectious centers in the usual manner.

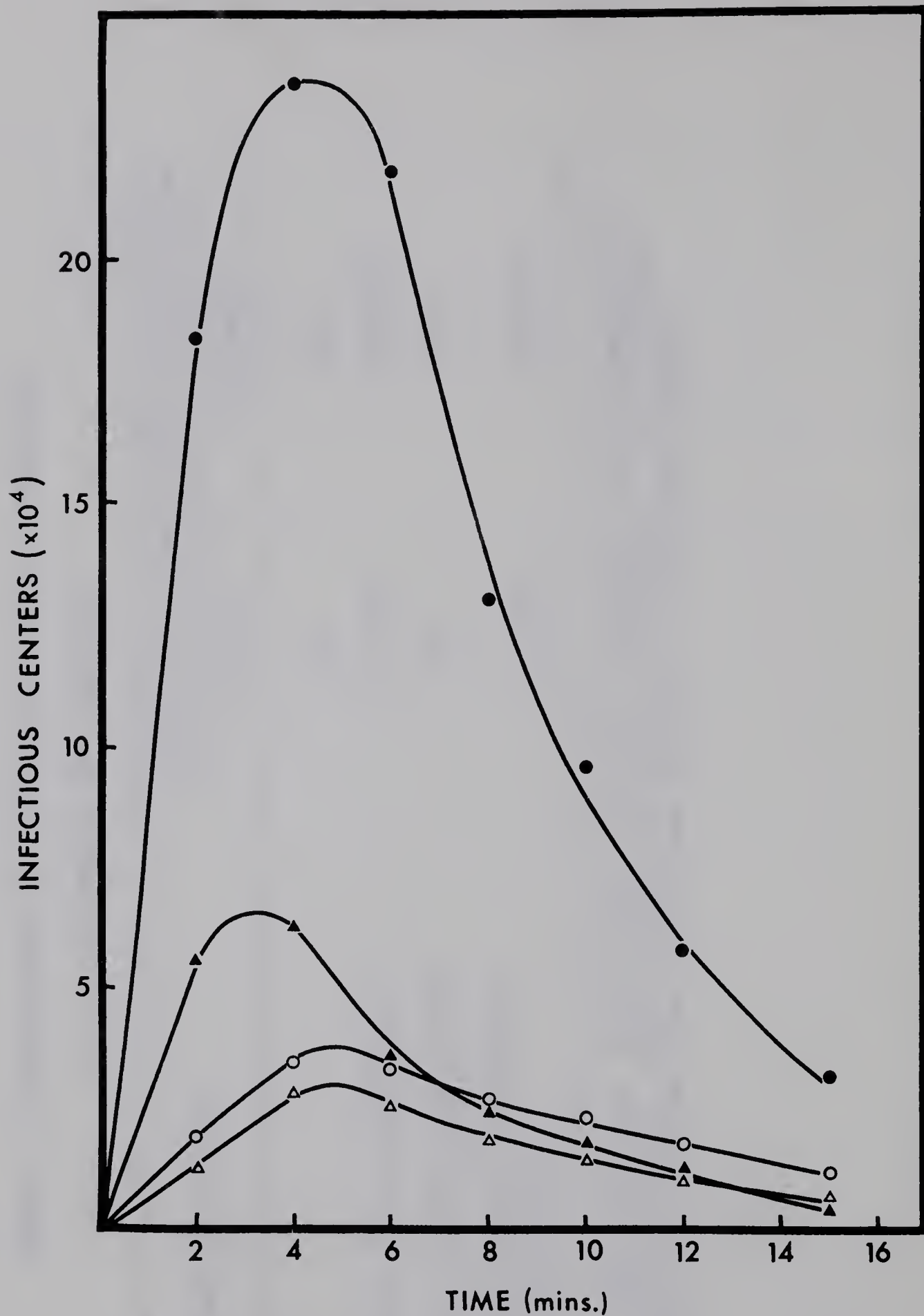


Figure 1.8. The effect of putrescine on infectious center formation in L cell-Mengo RNA mixtures incubated at 37°C in sucrose and sucrose-DMSO media. (○) 0.7 M sucrose/PBS, (●) 0.7 M sucrose/PBS containing 10% DMSO, (△) 0.7 M sucrose/PBS containing 0.03 M putrescine, (▲) 0.7 M sucrose/PBS containing 10% DMSO and 0.03 M putrescine.

1:20 and 1:40) of the stock viral RNA solution. It is quite clear that, in this system, putrescine has no effect whatsoever on the productive interaction of L cells and Mengo RNA. Other variations were examined in an attempt to explain Moscarello's data. For example, unwashed cells (presumably contaminated with extracellular nucleases) were used in the assay, as were media from which EDTA had been omitted (the thinking in this case being that putrescine may act as a chelating agent and thus stabilize the RNA). In each case, the results were clearly negative, - putrescine could not, under any of the conditions examined, be shown to stimulate the infectivity of the RNA.

It was observed, however, that when putrescine, at a final concentration of 0.04 M, was added to either of the incubation media, 0.6 M sucrose/PBS - 10% DMSO or PBS - DEAE-D, it caused a marked reduction in the sensitivity of the assay. This observation is illustrated by the data summarized in Figure 1.8 and Table 1.2. In each case, the values shown are the means of data obtained from two separate experiments.

Effect of Poly-L-ornithine on Infectious Center Formation

Koch et al. (1966) reported that they obtained more efficient infectious center formation in the polio RNA-HeLa cell system in the presence of poly-L-ornithine than was obtained by the addition of DEAE-D to the RNA-cell mixture. In the light of this claim, it was considered

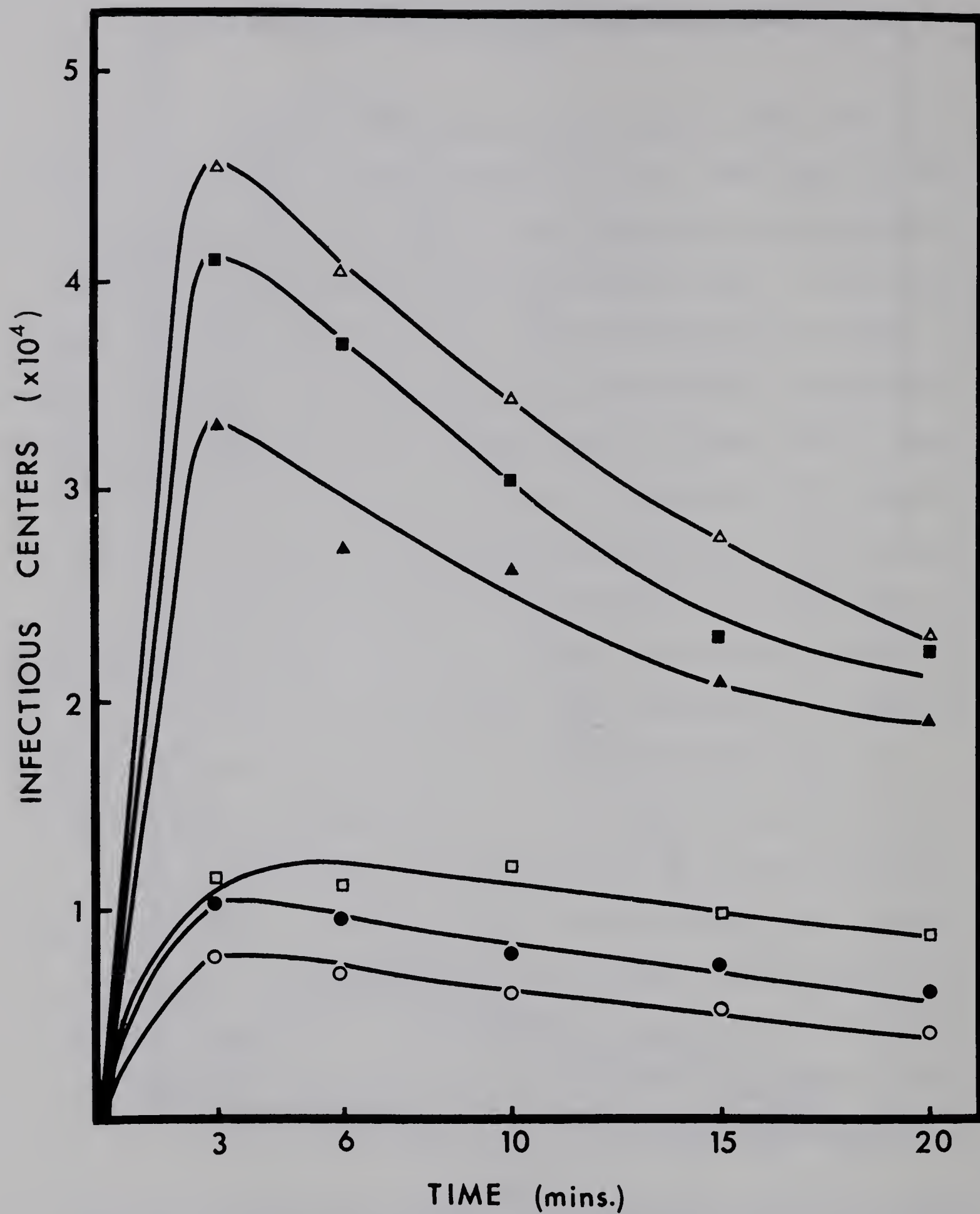


Figure 1.9. The effect of polyornithine (mol.wt. 21,000) concentration on infectious center formation in L cell-Mengo RNA mixtures incubated at 37° in PBS. (o) control (0 μg/ml), (●) 10 μg/ml, (□) 25 μg/ml, (■) 50 μg/ml, (Δ) 75 μg/ml, (▲) 100 μg/ml.

worthwhile to examine the effects of this polyamino acid in the L cell-Mengo RNA system.

In the first series of experiments, a suspended cell system, essentially identical to that used in the studies described in the preceding sections, and polyornithine of molecular weight 150,000 were used. In several experiments, no infectious centers whatsoever were obtained. This unexpected result led to an examination of the viability of L cells incubated in solutions of this polyornithine. The study showed clearly that only a very small percentage of the cells remained viable for more than a few minutes, even when the concentration of polyornithine in the suspending solution was as low as 1 μ g/milliliter. This matter is considered in more detail in Chapter 2 of this thesis (see Figures 2.16 and 2.17).

Subsequent to these experiments, polyornithine of a lower molecular weight (21,000) was obtained, and was examined in a similar fashion. It was found to be less toxic to L cells than the material of higher molecular weight, and, unlike the latter material, was shown to enhance the formation of infectious centers in the Mengo RNA-L cell system. Pooled data from a number of experiments are shown in Figure 1.9, from which it may be seen that the optimal concentration of the "low molecular weight" polyornithine was found to be 75 μ g/ml, and that at this concentration, a 6-fold stimulation in infectious

center formation (relative to that in PBS) was obtained. When one considers that the numbers of infectious centers produced in 0.6 M sucrose/PBS, 0.6 M sucrose/PBS - 10% DMSO and PBS-DEAE-D exceed the number produced in PBS by factors of 10-20, 50-100 and 400-1000, respectively, the stimulation given by polyornithine is rather unimpressive.

It should be noted that the usual assay procedure was modified slightly in the experiments illustrated by Figure 1.9. Koch et al. (1966) reported that maximum stimulation with polyornithine was obtained when the polycation was added to cells either before or after, but not at the same time as, the viral RNA. In agreement with that observation, it was found that fewer infectious centers were produced when Mengo RNA was assayed with polyornithine according to the usual procedure (in which the RNA is added to the complete incubation medium a few minutes before the cells are suspended in this solution) than were formed when the cells were suspended in PBS containing RNA and allowed to incubate for 5 minutes before the polyornithine was added. In contrast, maximum infectious center formation occurs with DEAE-D when the polycation is mixed with RNA prior to the addition of cells.

Agar Cell-Suspension Plaque Assay

An agar-cell suspension method for the assay of infectious viral RNA has been described (Koch et al., 1966; Koch and Bishop, 1968). In this method, cells in suspension

are incubated with RNA, after which the infected cells are plated with "indicator cells" as a thin layer in culture medium containing 0.6% agar ("soft agar"). Since the results summarized in the preceding section did not bear out the claims of Koch and his colleagues concerning the degree to which polyornithine stimulates infectious center formation, efforts were made to confirm their findings using the assay system that they described.

Before considering the data, it may be worthwhile to consider certain of the characteristics of this system that differ sharply from those of the suspended cell-monolayer system, and that are, in fact, very difficult to reconcile with much of the information gained from studies employing the latter technique. Koch and Bishop (1968), for example, reported that the number of cells in a fixed volume of RNA-incubation mixture can be varied over a wide range without exerting any significant influence on the observed titer of the RNA preparation. In contrast, Ellem and Colter (1960b) found the number of infectious centers formed in a fixed volume of a RNA solution to be directly proportional to the number of cells used. Also, Koch et al. (1966) incubated HeLa cell-polio RNA mixtures in PBS containing DEAE-D or polyornithine or both for 60 min at 37°. No data were presented to indicate how this time of incubation was chosen, but it seems a most unlikely one on at least two counts. First, the number of infectious centers formed in cell-RNA mixtures in the presence of

DEAE-D reaches a maximum in a very few minutes, after which the number decreases rather rapidly (see Figures 1.5 and 1.6 of this thesis). Secondly, polyornithine, as already pointed out, is highly toxic to cultured cells. In the light of these observations it would seem at least doubtful that a high proportion of infected cells would survive an incubation period of 60 minutes.

Nonetheless, several attempts were made to assay Mengo RNA in L cells using the agar cell-suspension method. In order to follow the procedure described by Koch et al. (1966) as closely as possible, the concentrations of polyornithine and DEAE-D recommended by that group were used, although neither was as high as that which had been established in the present study as being optimal for infectious center formation. In repeated attempts to make the assay work, plaques were obtained only when a short period of time (1-2 min) elapsed between the addition of "indicator cells" to the test cells and the plating of the sample in soft agar. When "indicator cells" and agar were added essentially simultaneously to the test cells and the suspension was plated immediately, no plaques were ever obtained. This observation suggested that the plaques resulted from infection of the "indicator cells" and did not, in fact, provide an estimate of the number of infectious centers in the test cells. This suggestion is not unreasonable,

since it has been shown that infectious RNA is not significantly inactivated by an incubation at 37° for an hour (Ellem and Colter, 1961a) if EDTA is present, that all potentially infectious RNA molecules are not removed by incubation with a single cell sample (a proposition considered in some detail in the following section), and that the interaction between DEAE-D-RNA and cells is very rapid.

The interpretation enunciated above was supported by data obtained from two other experiments. Duplicate cell-RNA mixtures (suspended in PBS containing DEAE-D) were incubated at 37° for 60 min, after which an equal volume of growth medium (10% serum) was added to one sample and an equal volume of serum-free growth medium was added to the other. The contents of each tube were then added to preformed L cell monolayers, and the plates were incubated at 37° in a humidified atmosphere (5% CO_2 in air) for one hour before the medium was removed and the agar overlay added. Four to ten times as many plaques were produced by the sample which had been diluted in serum-free growth medium as by the one diluted in growth medium containing 10% serum. Since one might reasonably expect that infected cells (infectious centers) should not be susceptible to inactivation by ribonucleases present in the serum, these results suggested that at least some (and perhaps most) of the plaques obtained

with the sample diluted in serum-free medium were the result of infection of monolayer cells by RNA molecules (presumably complexed with DEAE-D).

In another, and more definitive, series of experiments, aliquots of a RNA solution (containing DEAE-D) were incubated at 37° in either PBS or PBS containing approximately 10^6 cells per milliliter. After a one-hour incubation, indicator cells suspended in growth medium (2% serum) were added to each sample and the number of infectious centers in each was determined by plating in soft agar. No significant difference in the number of plaques produced by the various samples was found, which suggests rather strongly that in this system, the plaques that are produced result from infection of the "indicator" cells.

In the light of these results, use of the agar-cell suspension method was abandoned. The suspended cell system originally described by Ellem and Colter (1960a) seems clearly to be more sensitive, more precise, simple to perform and to provide data more amenable to rational interpretation. It was used in all other studies described in this thesis.

Efficiency of the Assay

It was suggested by Ellem and Colter (1961a) that the low infectivity of viral RNA preparations (as compared to the corresponding viral suspensions) can be

accounted for by the low permeability of the cell membrane to this macromolecule, and that in most, if not all, assays of viral RNA only a very small percentage of the potentially infectious RNA molecules are taken up by the cells. Experiments bearing on this point have been carried out, and the results tend to support this premise.

The study involved incubating a series of cell samples (each containing the same number of cells) with a single aliquot of viral RNA solution. Cell sample number 1 was incubated with 5 ml of RNA solution, after which the suspension was centrifuged to sediment the cells. The supernatant was decanted into a tube containing cell sample number 2, which was resuspended, incubated for the same time as was cell sample number 1, etc. This procedure was repeated for a total of 5 cell samples. Each cell sample, after incubation in the RNA solution, was resuspended in growth medium and the number of infectious centers therein measured in the usual manner. Three incubation media, namely 0.6 M sucrose/PBS, 0.6 M sucrose/PBS containing 10% DMSO, and PBS containing 100 μ g DEAE-D/ml, were examined. The incubation time (at 37°) for each cell sample was that which had been found to be optimal for infectious center formation in the particular medium (i.e., 6 min for the sucrose media and 3 min for PBS-DEAE-D). The results are summarized in Figure 1.10. In plotting these data, the number of

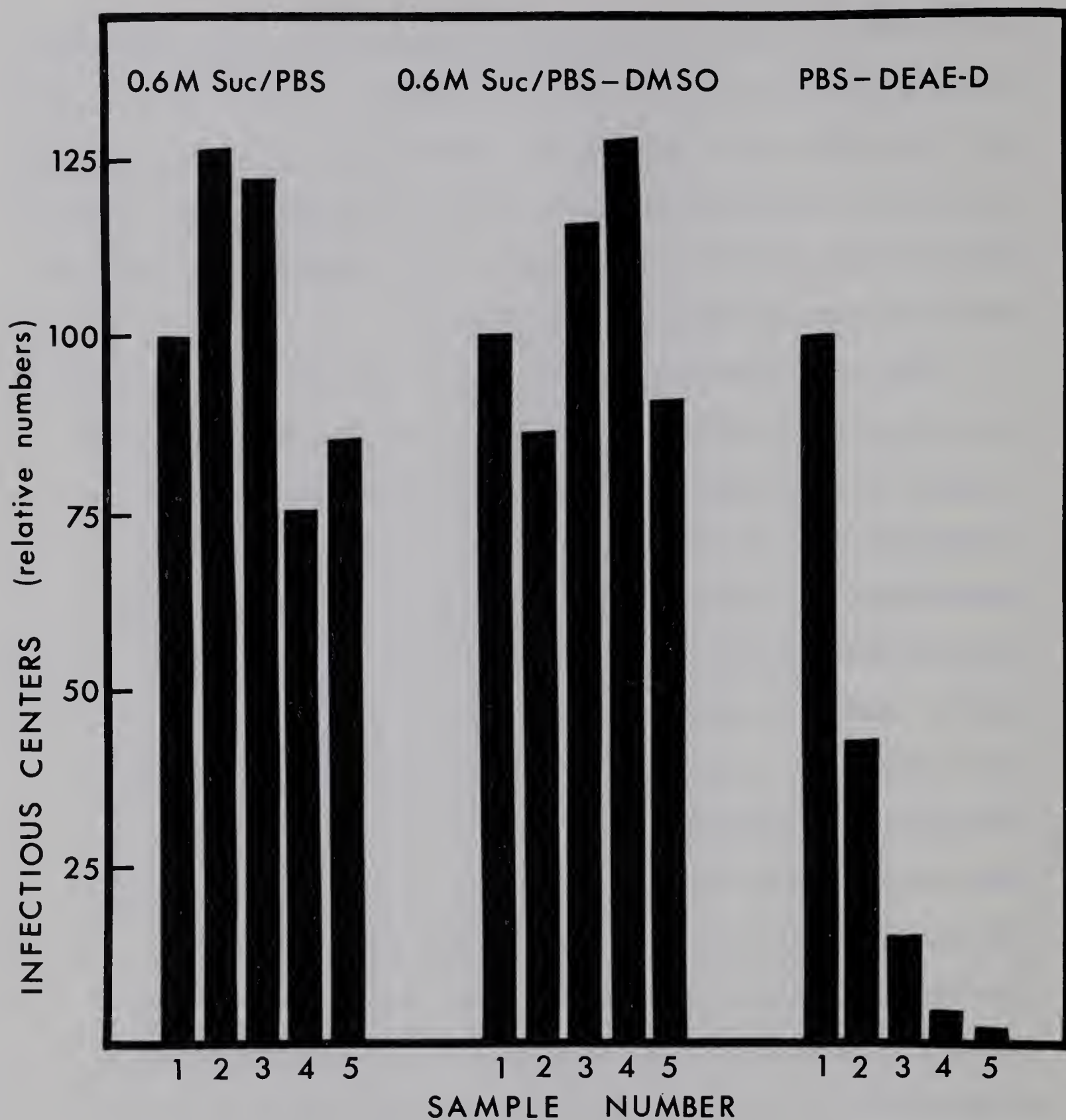


Figure 1.10. Infectious center formation in successive cell samples incubated with the same sample of RNA in the media indicated in the figure. The number of infectious centers produced in cell samples 2-5 is expressed as a percentage of the number produced in sample 1. See text for further details.

infectious centers produced in each of cell samples 2-5 was expressed as a percent of the number formed in the first. The values shown in Figure 1.10 are the means of values obtained from three separate experiments, in one of which two different concentrations of RNA (differing by a factor of 5) were used. The data obtained with the two concentrations of RNA were essentially identical.

When incubations were carried out in 0.6 M sucrose/PBS, with or without DMSO, it was found that essentially the same number of infectious centers were produced in each of the five cell samples, suggesting that the concentration of viral RNA was not reduced significantly even through five incubations with cells. The amount of scatter in the data may simply reflect the limits of reproducibility of the assay. In two similar experiments done earlier, some decrease in numbers of infectious centers formed in successive cell samples was observed. However, the fact that in these cases, as well as in the experiments that provided the data shown in Figure 1.10, a significant number of infectious centers were formed in each cell sample still provides strong support for the premise that only a very small percentage of the potentially infectious molecules are taken up by cells incubated with viral RNA in 0.6 M sucrose/PBS, with or without DMSO.

As illustrated in Figure 1.10, the picture obtained with

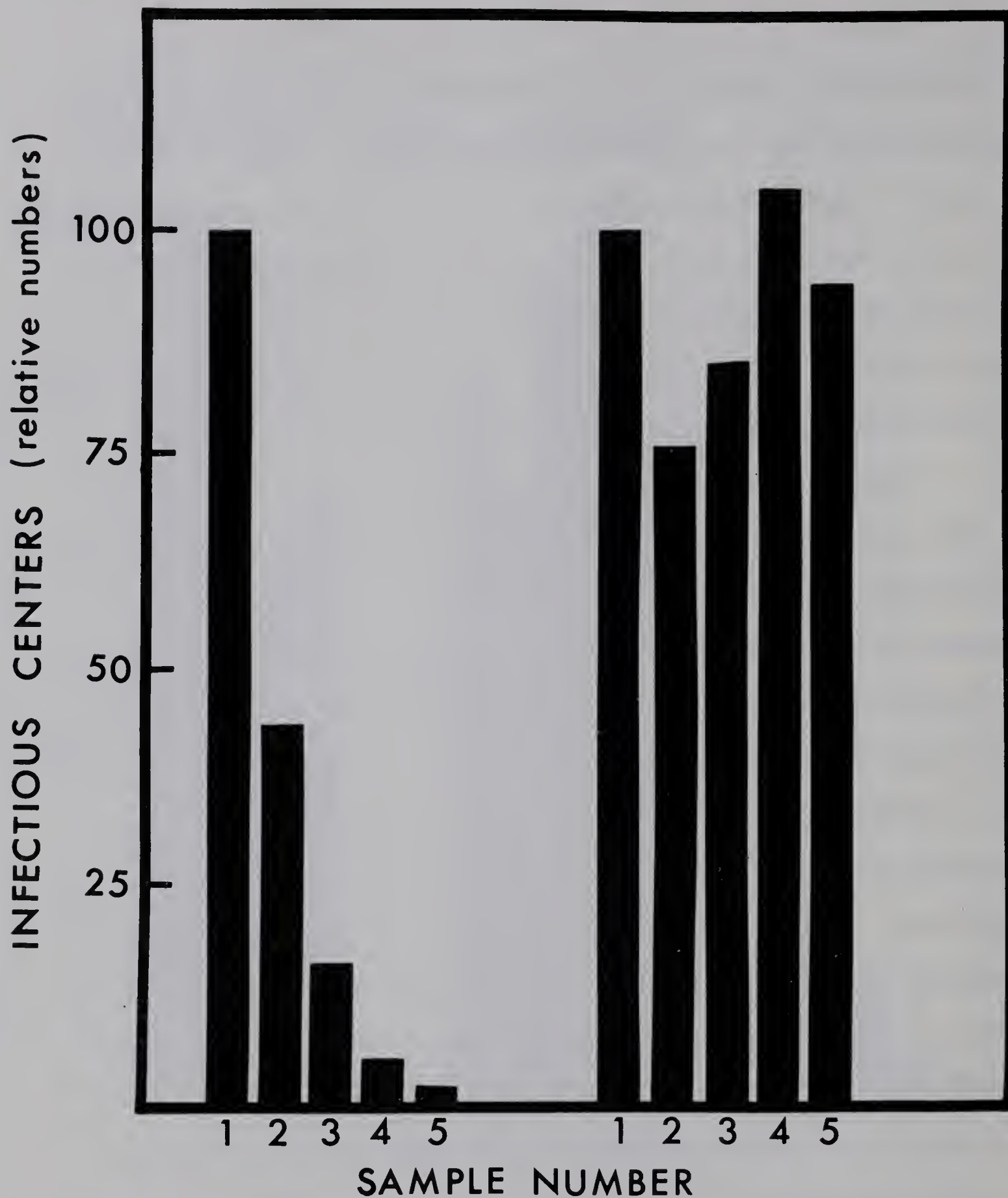


Figure 1.11. Infectious center formation in successive cell samples incubated with the same sample of RNA in PBS containing 100 μ g DEAE-D/ml. Additional DEAE-D (500 μ g) was added to samples 2-5 in the set which provided the data shown on the right of the figure. Data are expressed as indicated in the legend to Figure 1.10. See text for further details.

PBS containing DEAE-D was quite different. In all experiments, the number of infectious centers was found to drop very sharply in successive cell samples; the number formed in sample 5 in the experiments illustrated here being only 2% of the number formed in sample 1. This observation suggested that, in this medium, the RNA or DEAE-D or both were being effectively removed by the cells.

To resolve this question, the experiment (with PBS-DEAE-D as the incubation medium) was repeated, except that when each cell sample from 2 through 5 was resuspended in the supernatant from the preceding mixture, an additional 500 μg of DEAE-D were added. The results are shown in Figure 1.11. Under these conditions, there was no decrease in the number of infectious centers formed in five successive cell samples. If, as data presented earlier would suggest, 100 $\mu\text{g}/\text{ml}$ is close to the lower end of a range of concentrations of DEAE-D which gives optimal infectious center production, it is not surprising that, starting with that initial concentration, incubation with cells would remove enough DEAE-D from solution so that its concentration would be lowered to levels below those required for effective stimulation of infectious center formation. In fact, when the experiment was repeated with an RNA solution containing an initial concentration of DEAE-D of 500 $\mu\text{g}/\text{ml}$ and no further DEAE-D was added, as many infectious centers were formed in cell sample 5 as in the first sample.

Thus even with the most efficient of the assay media examined here, only a small percentage of the potentially infectious RNA molecules is detected, and it does not appear to be too reckless to suggest that this is true for other methods for the assay of viral RNA as well.

Discussion

It has been shown, in verification of earlier investigations, that more infectious centers are formed in L cell-Mengo RNA mixtures incubated in sucrose/PBS solutions than in PBS alone (with 0.6 M sucrose/PBS being the optimal medium in this series). It has been shown further that the addition of either DMSO or DEAE-D to the incubation medium (whether it be PBS or xM sucrose/PBS) stimulates the productive interaction between L cells and Mengo RNA, with DMSO being most effective when the basic incubation medium is 0.6 M sucrose/PBS, and DEAE-D exerting its maximum effect in PBS.

Amstey (1966) and Amstey and Parkman (1966) reported that they obtained maximum efficiency of infection of primary African green monkey kidney cells with polio RNA when the cells were exposed to RNA dissolved in buffered saline containing 40% DMSO. Solutions of elevated osmolarity containing DMSO were not tested. The present study has shown not only that many more infectious centers are formed when L cells are incubated with Mengo RNA in 0.6 M sucrose/PBS - 10% DMSO than when the incubation medium is

PBS-10% DMSO, but that the optimal concentration of DMSO in PBS as well as in sucrose/PBS solutions, is 10%. The differences between the results described here and those reported by Amstey and Parkman may simply reflect the different assay procedures used. Amstey and Parkman used monolayer cultures and a 50% tissue culture infectious dose ($TCID_{50}$) titration, while in the present work a suspended cell system and the more precise plaque titration was used. Also, and perhaps of equal significance, different cell types were employed. Quantitatively, DEAE-D was found to enhance the demonstrable infectivity of Mengo RNA to a much greater extent than DMSO. Unlike DMSO, DEAE-D was shown to be most effective in isotonic solution, although some stimulation was observed in hypertonic sucrose solutions. Vaheri and Pagano (1965) have studied the effects of DEAE-D on the assay of infectious polio RNA using HeLa cell monolayers. They reported that the infectivity of their RNA was approximately the same in PBS and in 0.8 M sucrose, and that DEAE-D stimulated the infectivity to about the same extent in the two media. In 1 M $MgSO_4$ solution, the infectivity of the polio RNA was much greater than in PBS or 0.8 M sucrose, but the addition of DEAE-D did not stimulate plaque formation, and, at higher concentrations of the polycation, even reduced the number of plaques formed.

In the present studies, the optimal concentration of DEAE-D was found to be of the order of 100-250 μg /milliliter.

This is in essential agreement with the findings of Koch et al. (1966), who reported that DEAE-D gave maximum stimulation in the polio RNA-suspended HeLa cell system at a concentration of 100-200 $\mu\text{g/ml}$. Dianzini et al. (1967) used the agar cell-suspension assay with Mengo RNA and L cells, and added DEAE-D, at a concentration of 800-1600 $\mu\text{g/ml}$, to the RNA solution. However, this solution was diluted 5-fold by mixing with the cell suspension, so that the final concentration in the cell-RNA incubation mixture was in the range of 160-320 $\mu\text{g/milliliter}$. In contrast, Bachrach (1966) found the optimal concentration of DEAE-D to be 1000 $\mu\text{g/ml}$ in the titration of FMD RNA on monolayers of calf kidney cells, and Vaheri and Pagano (1965), with the polio RNA-HeLa cell monolayer system, found that DEAE-D gave maximum stimulation at a concentration of 300-1000 $\mu\text{g/milliliter}$. These apparently conflicting findings may simply reflect either differences in the assay methods used (suspended cells versus monolayers for example), or differences in the level of resistance of the assay cells to damage at higher polycation concentrations. Certain preliminary studies carried out here have suggested that the optimal DEAE-D concentration may depend upon the total number or concentration of cells used in the assay, and also upon the concentration of RNA. However, definitive data concerning these points has not been obtained.

The finding that the addition of putrescine to the incubation medium (0.6 M sucrose/PBS) failed to stimulate

infectious center formation in L cell-Mengo RNA mixtures is in sharp disagreement with the report by Moscarello (1965), but is in agreement with the earlier observations of Smull and Ludwig (1962). There is no obvious way in which the data presented here and those published by Moscarello may be reconciled. It could perhaps be argued that since Moscarello used EMC-RNA, the systems used in the separate studies were different, but in view of the very close relationship between EMC and Mengo viruses, this argument seems rather untenable. At best, the report of Moscarello should probably be viewed with a certain scepticism.

The fact that putrescine actually inhibits infectious center formation when added to either the 0.6 M sucrose/PBS - 10% DMSO or PBS-DEAE-D system is not readily explained. However, Moscarello's report did include data that suggested that putrescine can bind to RNA, and the studies of Mahler and Mehrotra (1963) show that nucleic acids can interact with polyamines and diamines. As will be discussed in Chapter 3 of this thesis, an important factor in the stimulation of the infectivity of viral RNA by DEAE-D appears to be the binding of the polycation to RNA molecules. A reasonable explanation for the inhibition imposed by putrescine on infectious center formation in the PBS-DEAE-D system may be that by binding to RNA it interferes with the formation of the RNA-DEAE-D complexes. The basis of the inhibitory effect of putrescine in the 0.6 M sucrose/PBS - DMSO system is completely obscure.

Although some stimulation in infectious center production was observed in the presence of polyornithine (in the incubation medium PBS) it was much smaller than the stimulation reported by Koch et al. (1966) in the polio RNA-HeLa cell system (as high as 10^5), or even that observed by Dianzini et al. (1967) in the Mengo RNA-L cell system (10^2). These workers employed polyornithine of molecular weight 45,000 (which unfortunately could not be obtained for examination) while the polymer of molecular weight 21,000 was used in the present study. Pagano et al. (1967) found that with DEAE-D's of different molecular weights, the efficiency with which the compound stimulated the infectivity of polio RNA decreased as the molecular weight decreased. It is possible that a similar situation holds true for polyornithines of different molecular weights, although the fact that no infectious centers could be detected when incubations were carried out in the presence of the polyornithine of molecular weight 150,000 would seem to argue against it. On the other hand, of the two polyornithines studied, the larger molecule was found to be much more toxic to cells than was the smaller, and it is conceivable that both ability to stimulate cell-RNA interaction and toxicity to cells increase with increasing molecular size of the polycation. If this were so, one could imagine that optimal infectious center production (reflecting some sort of balance between stimulation and toxicity) would be obtained

with a molecule of intermediate size (perhaps 45,000). Even making these assumptions, however, much of the procedure described by Koch et al. would appear to be something of a fruitless exercise. Data presented in this chapter indicate clearly that a great many, if not all, of the infectious centers measured by their method are produced in the "indicator" rather than the test cells.

Direct evidence in support of the premise that presently available assay procedures detect only a small proportion of the potentially infectious molecules in a preparation of viral RNA has been obtained from the studies in which successive cell samples were incubated in a single RNA solution. It should be pointed out, however, that other investigators have claimed otherwise. Holland et al. (1960) reported that while only a small fraction of P^{32} -labelled polio RNA was adsorbed to HeLa cell monolayers, very little infectivity could be detected in the supernatant solution, and suggested as a possible explanation that only a relatively small fraction of the molecules were infectious. Sprunt et al. (1967) found that, in hypertonic solution, 75% of infectious RNA units were adsorbed to cell monolayers within 1 minute. The absence of demonstrable infectivity in supernatants drained from monolayers was interpreted by these investigators as evidence for very efficient attachment of infectious RNA to the cells. Alternate explanations seem more reasonable. It is likely

that much of the unabsorbed RNA would be degraded (data on this point is presented in Chapter 3 of this thesis), and it is doubtful that 0.1 ml of RNA solution could be recovered from a monolayer with great efficiency. Koch (1960) has, in fact, successfully recovered infectious RNA from cell monolayers, and found that the residual infectivity was considerably enhanced by restoring the hypertonicity of the solution. Bases and Huppert (1966) incubated cells and labelled viral RNA in a medium of physiological ionic strength. They claimed that virtually all the infectivity became cell associated in about 1 minute, although most of the isotope (90% of which was in the form of small, degraded molecules) could be recovered from the supernatant.

That at least some of the viral RNA molecules exposed to L cells in the suspended cell system used in these studies become degraded will be shown in Chapter 3 of this thesis. However, experiments described in the present chapter show clearly that much of the infectious RNA does remain in solution after incubation with cells in either sucrose media or in PBS-DEAE-D. Thus, even though a fraction of the RNA molecules become degraded to smaller, non-infectious molecules during incubation, some feature(s) of the assay other than any lack of potentially infectious RNA molecules limits the number of infectious centers formed. Some of these will be discussed further in the following chapters.

CHAPTER 2

The Interaction of Mengo RNA with L, HeLa and Human Diploid Cells (a Comparative Study)

Introduction

Although a few investigators have assayed a single viral RNA species in cells of more than one type (including, in some cases, cell types which are susceptible as well as those which are not susceptible to infection by the intact virus) very few, if any, definitive studies designed to define the optimal conditions for the interaction of a single RNA species with a variety of cell types, have been made. A study of this kind has been carried out as a part of the broader investigation described in this thesis. The interaction of Mengo RNA with HeLa and human diploid cells was examined, and the optimal conditions for the interaction in various media and for each cell type was established. Using this information, and that obtained from the comparable study with L cells described in Chapter 1, a direct comparison was made of infectious center formation in Mengo RNA - (L, HeLa or human diploid) cell mixtures in a variety of incubation media. The results of the study are summarized in this chapter, and the differences in the conditions which are optimal for productive cell-RNA interaction in the case of these three cell types are discussed.

Materials and Methods

Growth of Cells

HeLa cells were grown in both monolayer and suspension cultures, employing the growth and spinner media described in the Routine Materials and Methods section, except that horse serum was replaced by calf serum. Cells were grown in 1-liter Blake bottles until confluent monolayers (containing approximately 50×10^6 cells) were formed, and were then detached from the glass by treatment with trypsin and transferred to spinner flasks. The procedure followed was identical to that used for the growth and handling of L cells (see Chapter 1).

Human diploid cells. The strain of diploid cells used in this study was developed by Miss Elizabeth Garbutt of this department from infant foreskin tissue obtained from the University of Alberta Hospital. It was established by chromosomal analysis that the cell strain was diploid.

Cells were maintained in monolayer culture in a growth medium consisting of Earle's balanced salt solution (Earle, 1943) containing double the concentration of nutrients recommended by Eagle (1955), 10% fetal calf serum, 5% inactivated calf serum ("inactivated" by heating at 56° for 30 min), aureomycin (final concentration = $50 \mu\text{g/ml}$) and Bacto-peptone (Difco Laboratories, Detroit, Mich.; final concentration = 0.25%).

Titration of RNA

HeLa cells were collected from spinner cultures by centrifugation and washed three times in PBS containing 0.05% BPA. Diploid cells were collected from Blake bottles by treatment with 0.5% trypsin in Hanks' salt solution (5 min at room temperature followed by 15 min at 37°) and were washed twice in PBS containing 0.05% BPA. They were then resuspended in PBS containing 0.05% BPA and allowed to stand at 4° for 2 hrs, at which time cell counts and viabilities were measured, and the required number of cells was collected by centrifugation.

The RNA assay procedure was the same as that described for the L cell system. Cells were suspended at a concentration of 2.5×10^6 cells/ml in the prewarmed (37°) incubation medium containing Mengo RNA, and the suspension was placed in a water bath at 37°. At intervals 1 ml aliquots were removed and added to 9 ml growth medium. Further dilutions were made when necessary, and aliquots of the suspensions were added to monolayers of L cells in order to quantitate the numbers of infected cells present. The procedure was identical in all respects to that described for the assay of RNA in L cells by the suspended cell system. It should, perhaps, be emphasized that it was necessary to titrate the infectious centers formed in HeLa and human diploid cells on indicator monolayers of L cells because neither HeLa nor human diploid cells can be infected with intact Mengo virus. This being the case, plaques would

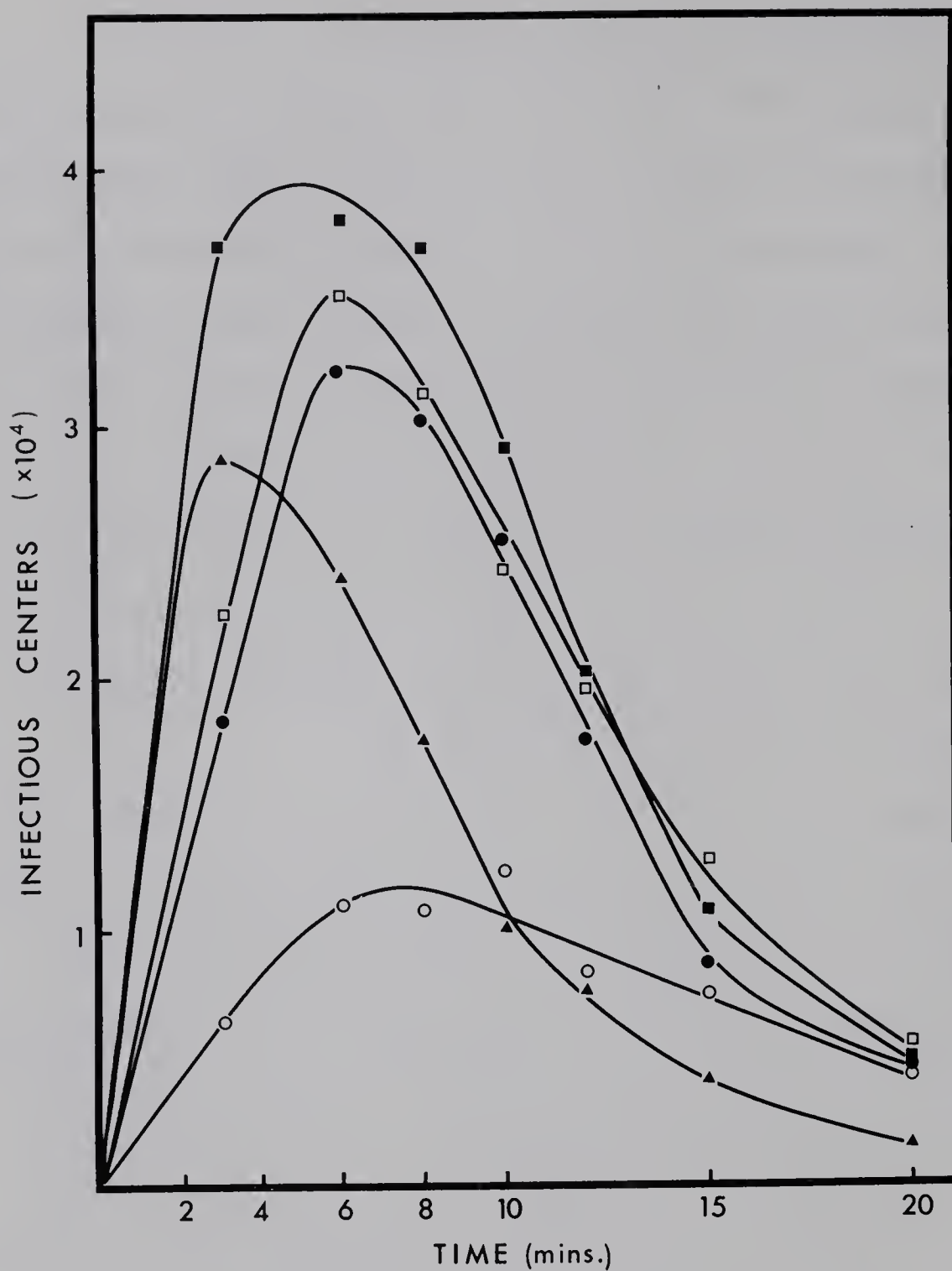


Figure 2.1. Infectious center formation in HeLa cell-Mengo RNA mixtures incubated at 37°C in (○) 0.8 M NaCl, (●) 1.0 M NaCl, (□) 1.2 M NaCl, (■) 1.4 M NaCl, (▲) 1.6 M NaCl. All solutions were buffered at pH 7.4 with 0.02 M Na phosphate buffer.

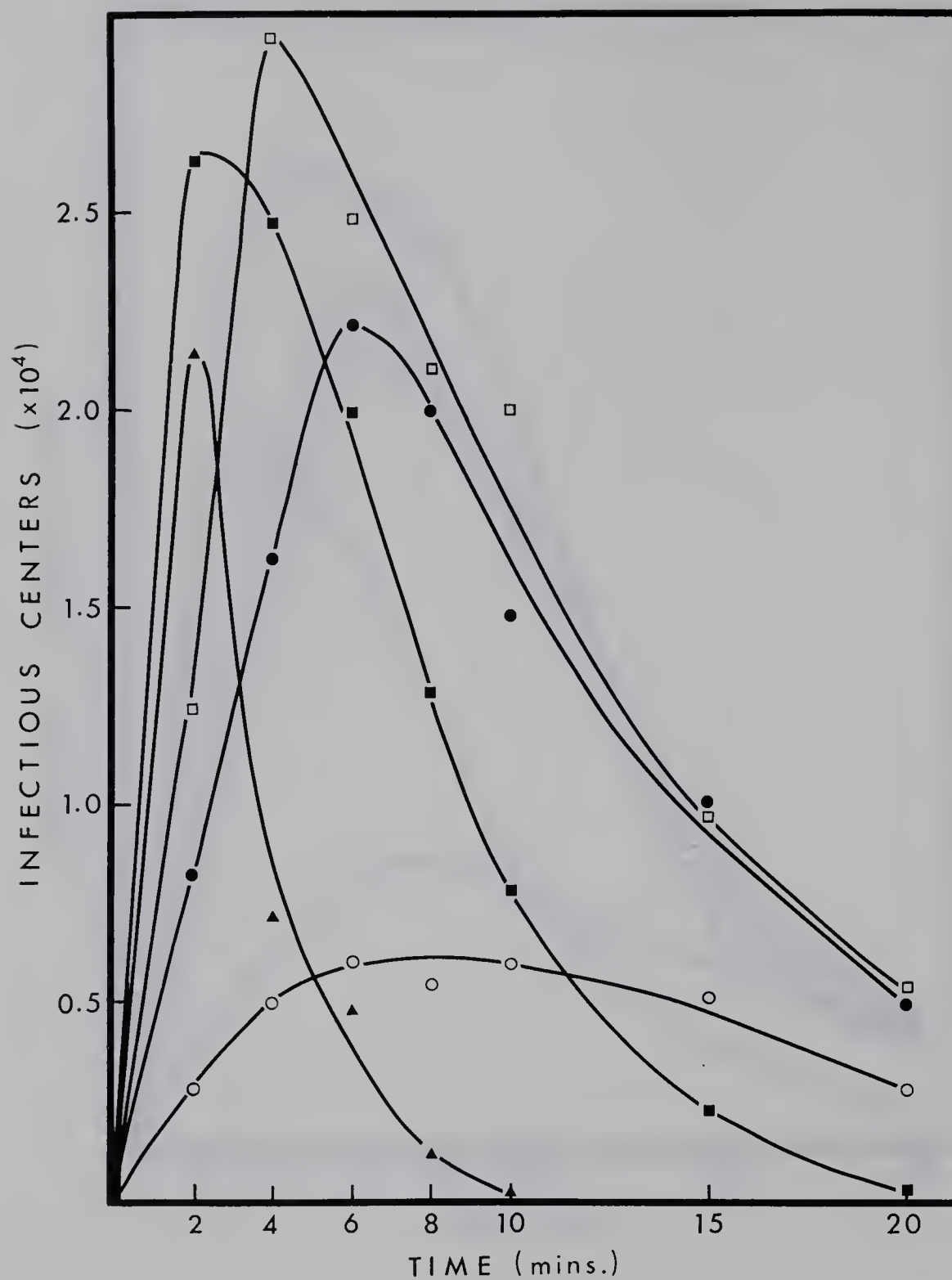


Figure 2.2. Infectious center formation in HeLa cell-Mengo RNA mixtures incubated at 37°C in (○) 0.8 M sucrose/PBS, (●) 1.0 M sucrose/PBS, (□) 1.2 M sucrose/PBS, (■) 1.4 M sucrose/PBS, (▲) 1.6 M sucrose/PBS.

not have been produced had the suspensions of treated HeLa or human diploid cells been plated on monolayers of the homologous cell type.

Results

Infection of HeLa Cells with Mengo RNA

Interaction in hypertonic NaCl and sucrose/PBS media.

The interaction of poliovirus RNA with HeLa cells in suspension was studied previously by Ellem and Colter (1961a), who measured infectious center formation in (a) a series of phosphate-buffered (0.02 M, pH 7.3) NaCl solutions (x M PBS) and (b) a series of solutions of sucrose in PBS (x M sucrose/PBS). They found that, in these two series of media, maximum numbers of infectious centers were produced in 0.9 M PBS and 0.98 M sucrose/PBS respectively, with the efficiency of infection being considerably higher in the former than in the latter.

As a point of departure for the present study, a careful examination of infectious center formation in Mengo RNA-HeLa cell mixtures in the same two series of media was undertaken. In the hypertonic saline series, the range of NaCl concentration from 0.8 to 1.6 M was employed, and sucrose concentrations covering the same range were included in the sucrose/PBS series. The pertinent observations, illustrated by Figures 2.1 and 2.2, are that the optimal media for infectious center formation in the x M

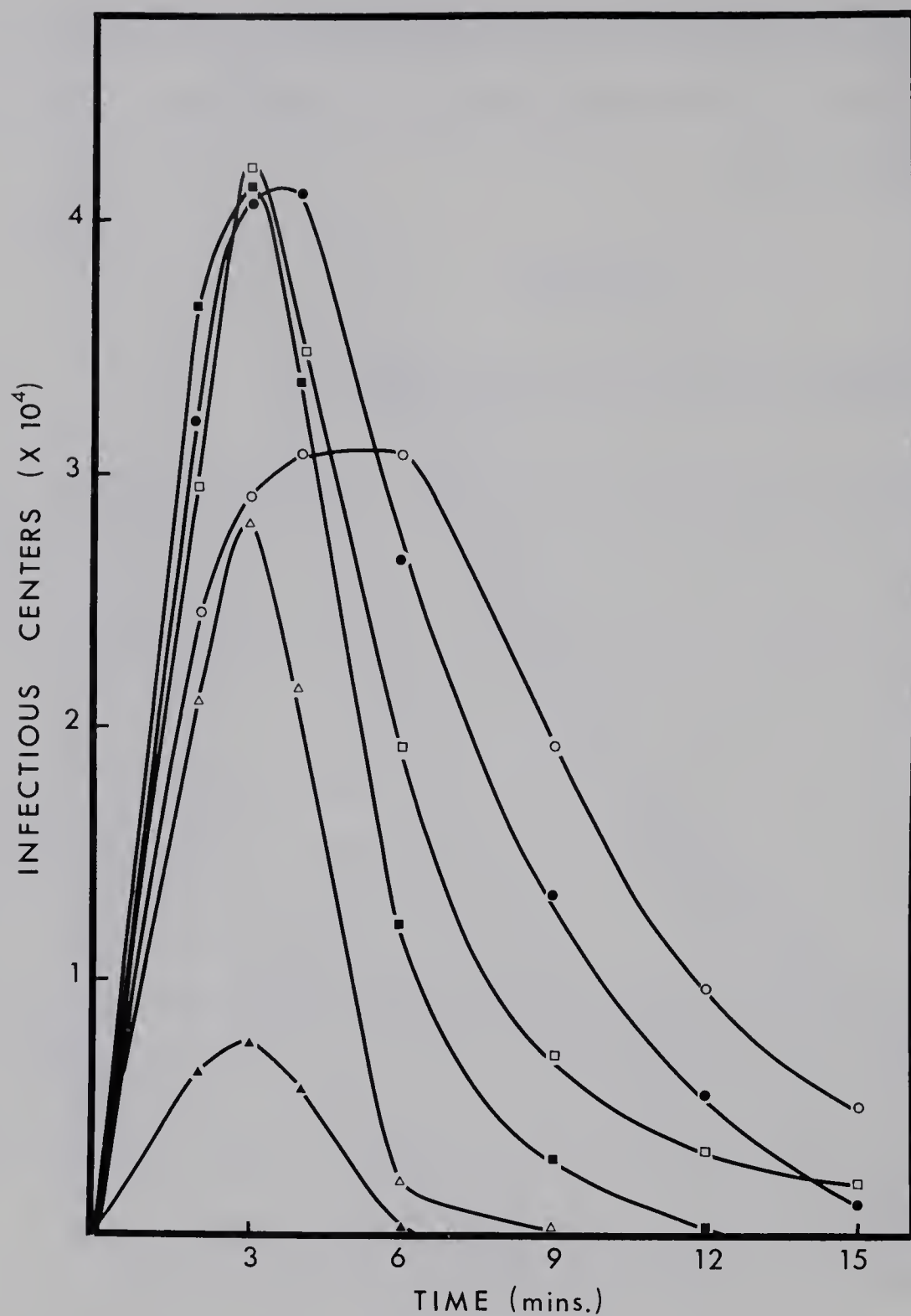


Figure 2.3. The effect of DMSO concentration on the formation of infectious centers in HeLa cell-Mengo RNA mixtures incubated at 37°C in 1.2 M sucrose/PBS. (○) 5% DMSO, (●) 7.5% DMSO, (□) 10% DMSO, (■) 12.5% DMSO, (△) 15% DMSO, (▲) 20% DMSO.

PBS and x M sucrose/PBS series of media were found to be 1.4 M PBS and 1.2 M sucrose/PBS respectively.

These optima differ significantly from those reported by Ellem and Colter for the poliovirus RNA-HeLa cell system, and there is no obvious, rational explanation for the differences. It seems unlikely that they can be explained by the fact that different species of RNA were used in the separate studies (Mengo and poliovirus RNA's are very similar with respect to physical and chemical parameters). A more likely explanation is that the differences reflect the fact that different lines of HeLa cells were used in the two studies, and that the cells were handled somewhat differently prior to incubation with the viral RNA (recall that the presence of a low concentration of BPA in the PBS wash solution was shown to protect the cells).

Effect of DMSO. Since, in the x M sucrose/PBS series, maximum infectious center formation was found to occur in 1.2 M sucrose/PBS, this solution was used initially as the basic incubation medium in studies aimed at determining the optimal DMSO concentration. The results of several successive experiments provided the data shown in Figure 2.3. Clearly, there is no significant difference between the numbers of infectious centers formed in cell-RNA mixtures incubated in 1.2 M sucrose/PBS containing 7.5%, 10% or 12.5% DMSO. This being the case, DMSO at a

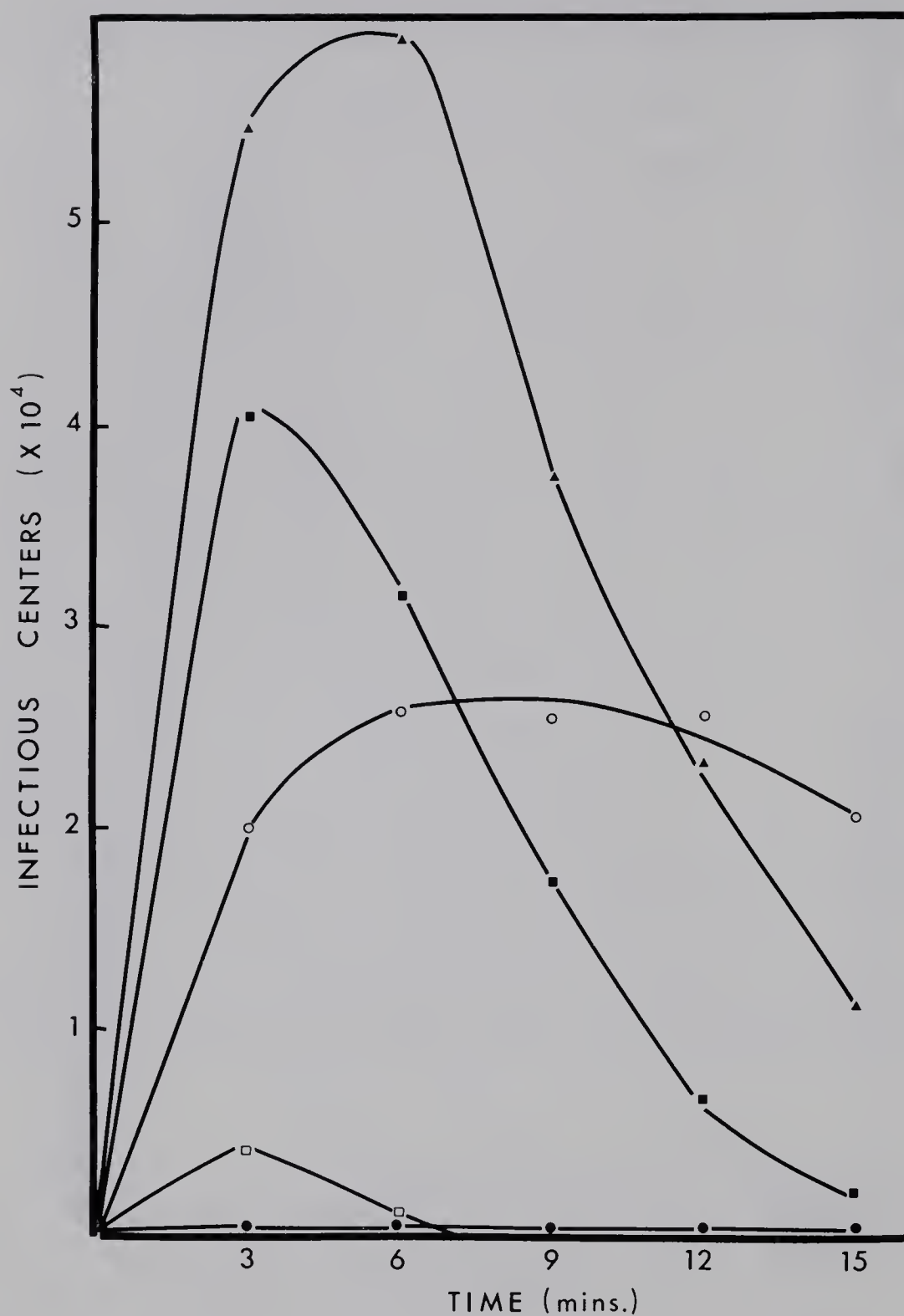


Figure 2.4. Infectious center formation in HeLa cell-Mengo RNA mixtures incubated at 37° in (●) PBS, (○) 0.4 M sucrose/PBS, (▲) 0.8 M sucrose/PBS, (■) 1.2 M sucrose/PBS, (□) 1.6 M sucrose/PBS. All media contained 10% DMSO.

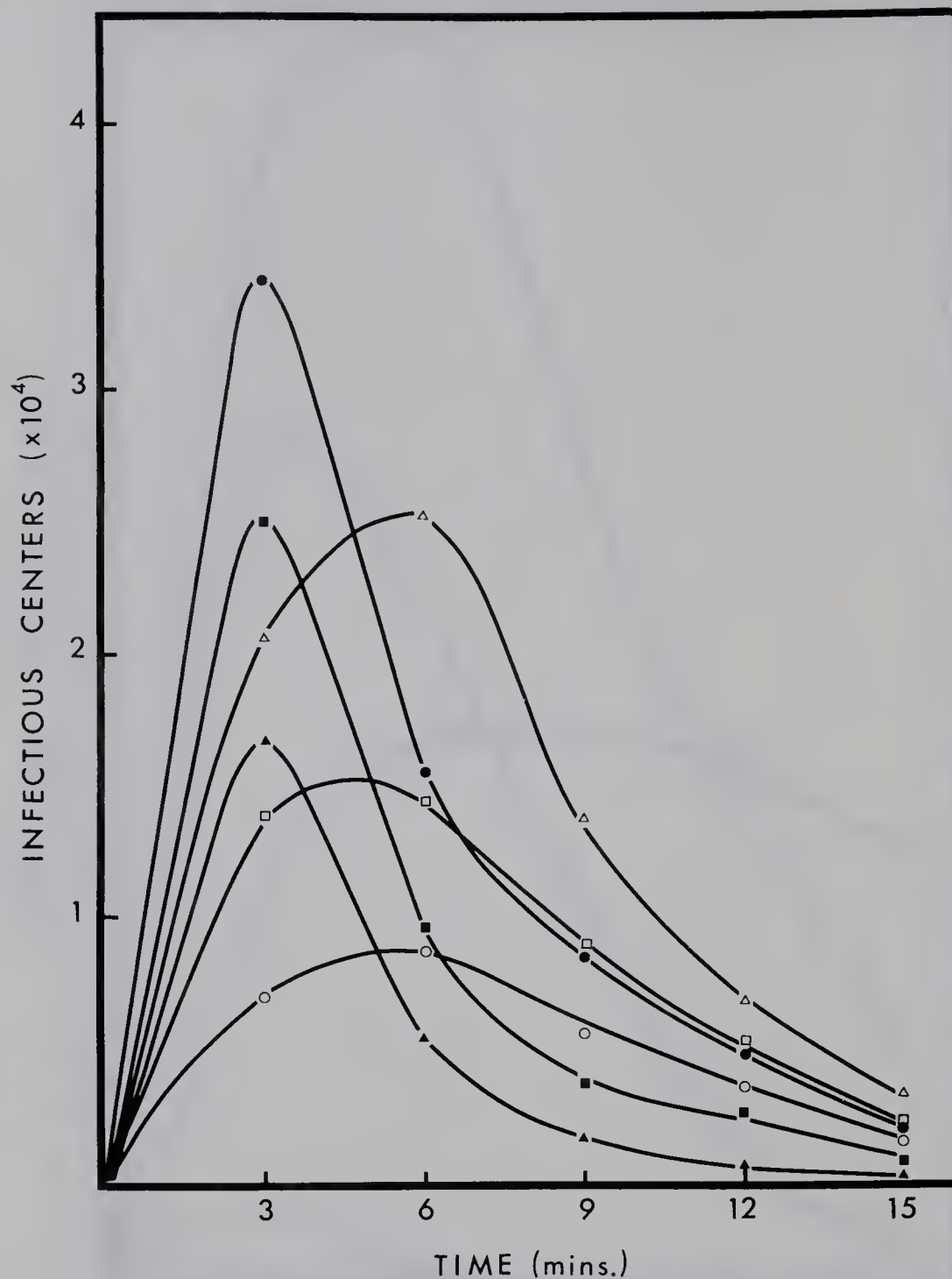


Figure 2.5. Infectious center formation in HeLa cell-Mengo RNA mixtures incubated at 37°C in (○) 0.8 M sucrose/PBS, (●) 0.8 M sucrose/PBS-10% DMSO, (□) 1.0 M sucrose/PBS, (■) 1.0 M sucrose/PBS-10% DMSO, (△) 1.2 M sucrose/PBS, (▲) 1.2 M sucrose/PBS-10% DMSO.

concentration of 10% was used in all subsequent experiments (as it was in the L cell system).

The possibility that the combination of 1.2 M sucrose and 10% DMSO may not be optimal for the productive interaction between Mengo RNA and HeLa cells was considered next. To do so, a series of sucrose solutions (x M sucrose/PBS) containing 10% DMSO were used as incubation media. It was found that the maximum number of infectious centers was produced in 0.8 M sucrose/PBS (containing 10% DMSO). Figure 2.4 illustrates this observation, and also shows clearly that RNA interacts much more effectively with HeLa cells in sucrose/PBS-DMSO solutions than in PBS containing DMSO. Subsequently, other experiments were done which established that in 0.8 M sucrose/PBS, as well as in 1.2 M sucrose/PBS, 10% is the optimal concentration of DMSO.

Finally, the overall stimulation that may be obtained by the addition of DMSO to sucrose/PBS media was estimated by measuring the number of infectious centers formed in 0.8-, 1.0- and 1.2 M sucrose/PBS solutions and in the same solutions containing 10% DMSO. The results of this study are summarized in Figure 2.5. As was observed earlier (Figures 2.2 and 2.4, respectively), (a) in the absence of DMSO, the number of infectious centers formed increases as the sucrose concentration of the incubation medium increases from 0.8 M to 1.2 M, and (b) in the presence of 10% DMSO, the number of infectious centers formed decreases

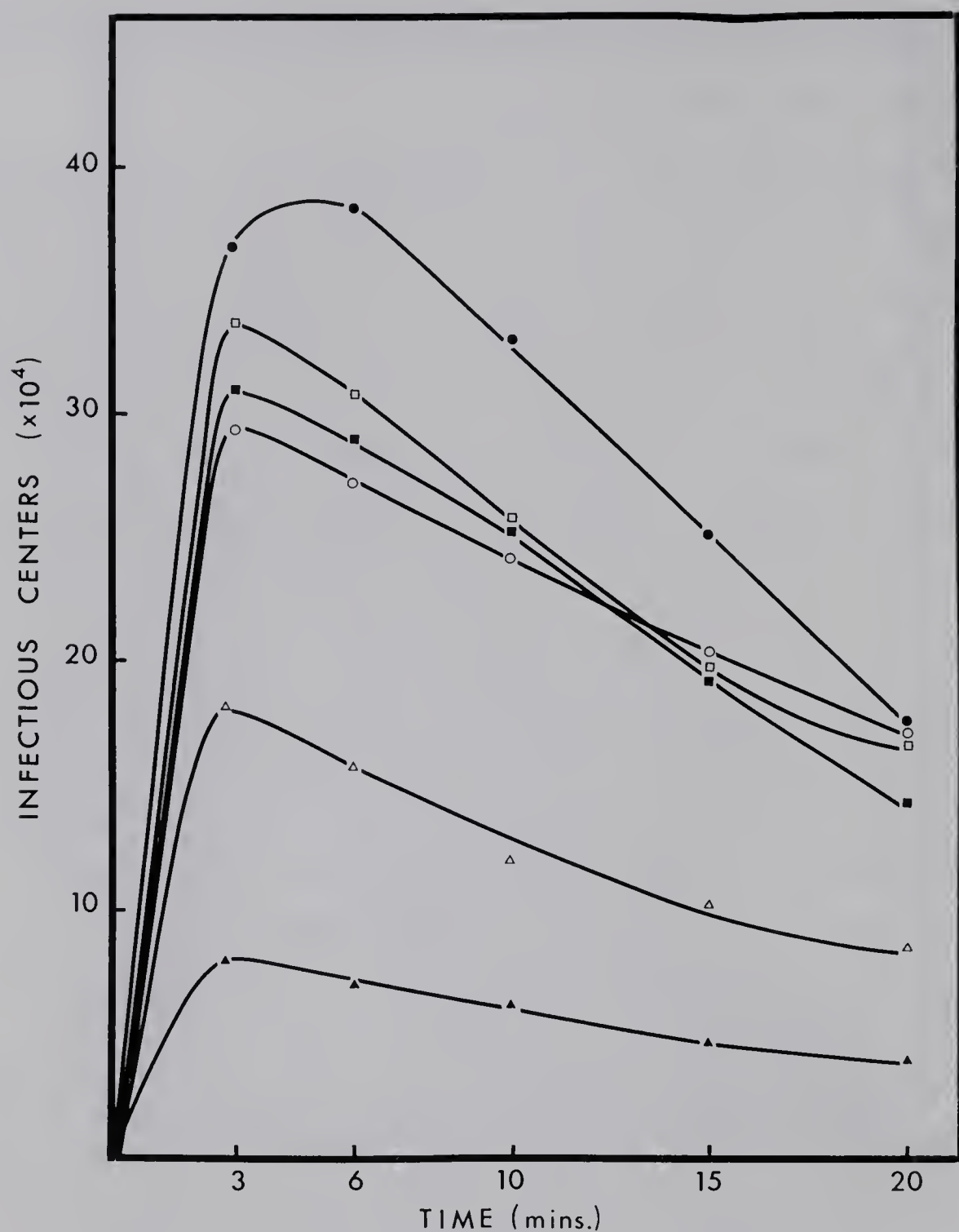


Figure 2.6. The effect of DEAE-D concentration on the formation of infectious centers in HeLa cell-Mengo RNA mixtures incubated at 37°C in PBS. (○) 50 µg/ml, (●) 100 µg/ml, (□) 250 µg/ml, (■) 500 µg/ml, (△) 1000 µg/ml, (▲) 1500 µg/ml.

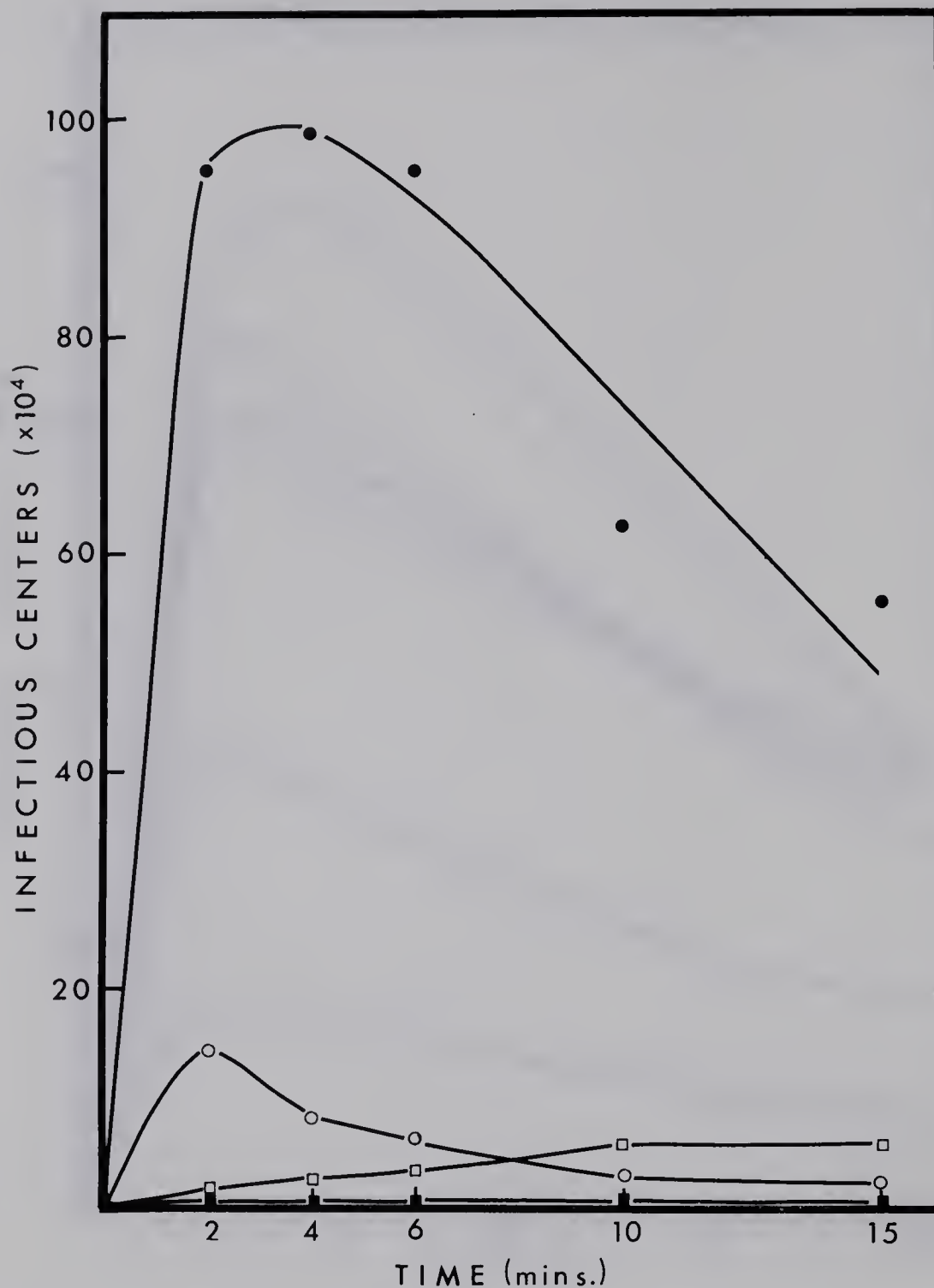


Figure 2.7. Infectious center formation in HeLa cell-Mengo RNA mixtures incubated at 37°C in (○) 0.05 M NaCl, (●) 0.14 M NaCl, (□) 0.3 M NaCl, (■) 0.6 M NaCl. All media contained 100 µg DEAE-D/ml, and were buffered at pH 7.4 with 0.02 M Na phosphate buffer.

as sucrose concentration increases from 0.8 M to 1.2 M. In fact, fewer infectious centers are produced in 1.2 M sucrose/PBS containing 10% DMSO than in 1.2 M sucrose/PBS, although the presence of 10% DMSO in 0.8 M sucrose/PBS results in a 4-fold stimulation in infectious center formation. Overall, 0.8 M sucrose/PBS containing 10% DMSO is only marginally better than 1.2 M sucrose/PBS with respect to stimulating infectious center formation in Mengo RNA-HeLa cell mixtures.

Effect of DEAE-D. The addition of DEAE-D to HeLa cell-Mengo RNA mixtures (in PBS) was found to produce a marked stimulation in infectious center formation. The results obtained from a series of experiments designed to determine the optimal concentration of DEAE-D in the HeLa cell system were similar to those obtained from earlier studies of the L cell system, and are illustrated in Figure 2.6. Although the maximum number of infectious centers was found at a DEAE-D concentration of 100 $\mu\text{g/ml}$, the stimulations observed at concentrations of 50, 250 and 500 $\mu\text{g/ml}$ were not dramatically less.

That DEAE-D is most effective when added to a basic solution of physiological ionic strength is shown clearly by the data given in Figure 2.7. At NaCl concentrations above and below 0.14 M (isotonic), infectious center formation in the presence of 100 $\mu\text{g DEAE-D/ml}$ was found to be sharply inhibited.

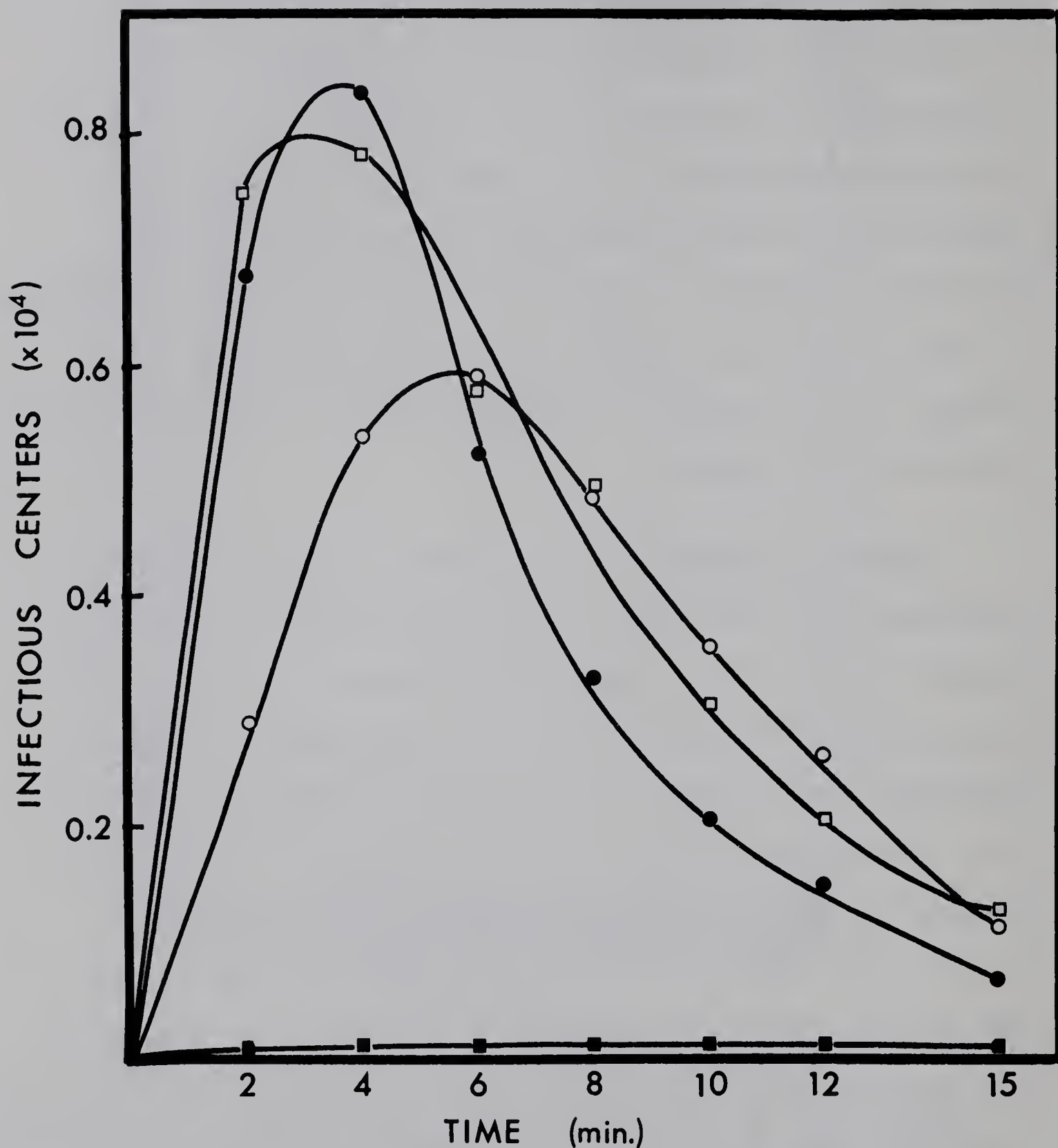


Figure 2.8. Infectious center formation in HeLa cell-Mengo RNA mixtures incubated at 37°C in (■) PBS, (□) 1.4 M NaCl (phosphate buffered), (○) 1.2 M sucrose/PBS, (●) 0.8 M sucrose/PBS containing 10% DMSO.

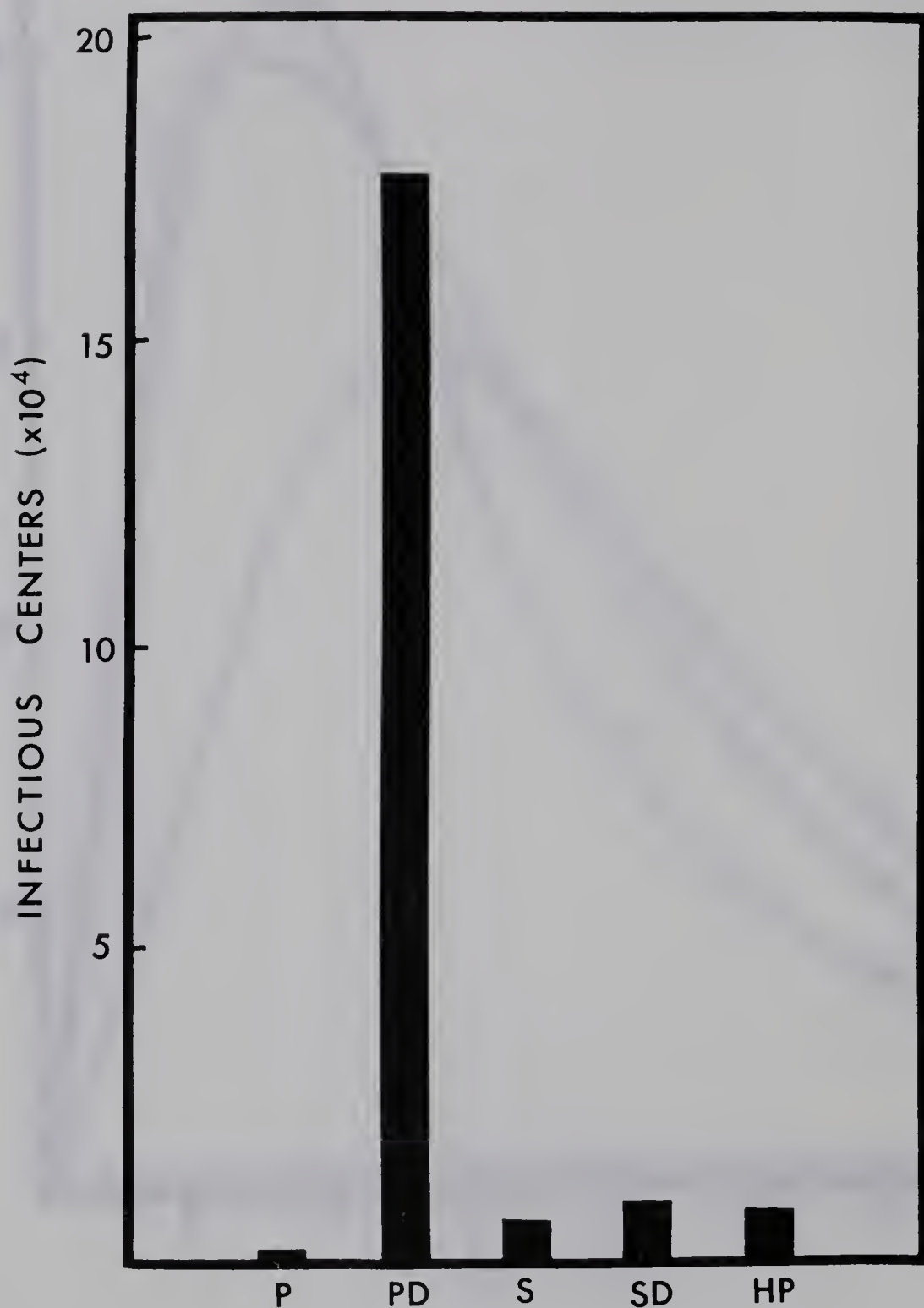


Figure 2.9. A comparison of infectious center formation in HeLa cell-Mengo RNA mixtures incubated at 37° in (P) PBS, (PD) PBS containing 100 μ g DEAE-D/ml, (S) 1.2 M sucrose/PBS, (SD) 0.8 M sucrose/PBS containing 10% DMSO, (HP) 1.4 M PBS.

A comparison of infectious center formation in PBS and the optimal hypertonic NaCl, sucrose/PBS, sucrose/PBS-DMSO and PBS-DEAE-D media. Infectious center formation in HeLa cells incubated with Mengo RNA in five different media was measured, and the data obtained from several separate experiments are summarized in the next two figures. In these experiments, aliquots were removed from each cell-RNA mixture at 2 minute intervals, and assayed for infectious centers. The data obtained from determinations of infectious center formation in 0.14 M PBS, 1.4 M PBS, 1.2 M sucrose/PBS, and 0.8 M sucrose/PBS - 10% DMSO are shown in Figure 2.8. The number of infectious centers formed in each of the last three media was found to be much greater than the number produced in PBS (less than 100 infectious centers/ 10^6 cells/ml undiluted RNA with this particular RNA preparation). The efficiency with which HeLa cells were infected by Mengo RNA was shown to be somewhat lower in 1.2 M sucrose/PBS than in either 1.4 M PBS or in 0.8 M sucrose/PBS containing 10% DMSO, which proved to be equally effective in stimulating infectious center formation. A summary of infectious center formation in all five incubation media is given in Figure 2.9. It should be noted that in compiling the data summarized here, the same dilution of a single RNA preparation was used in all incubation media, and that the value shown for each medium is the maximum number present at any time in that medium

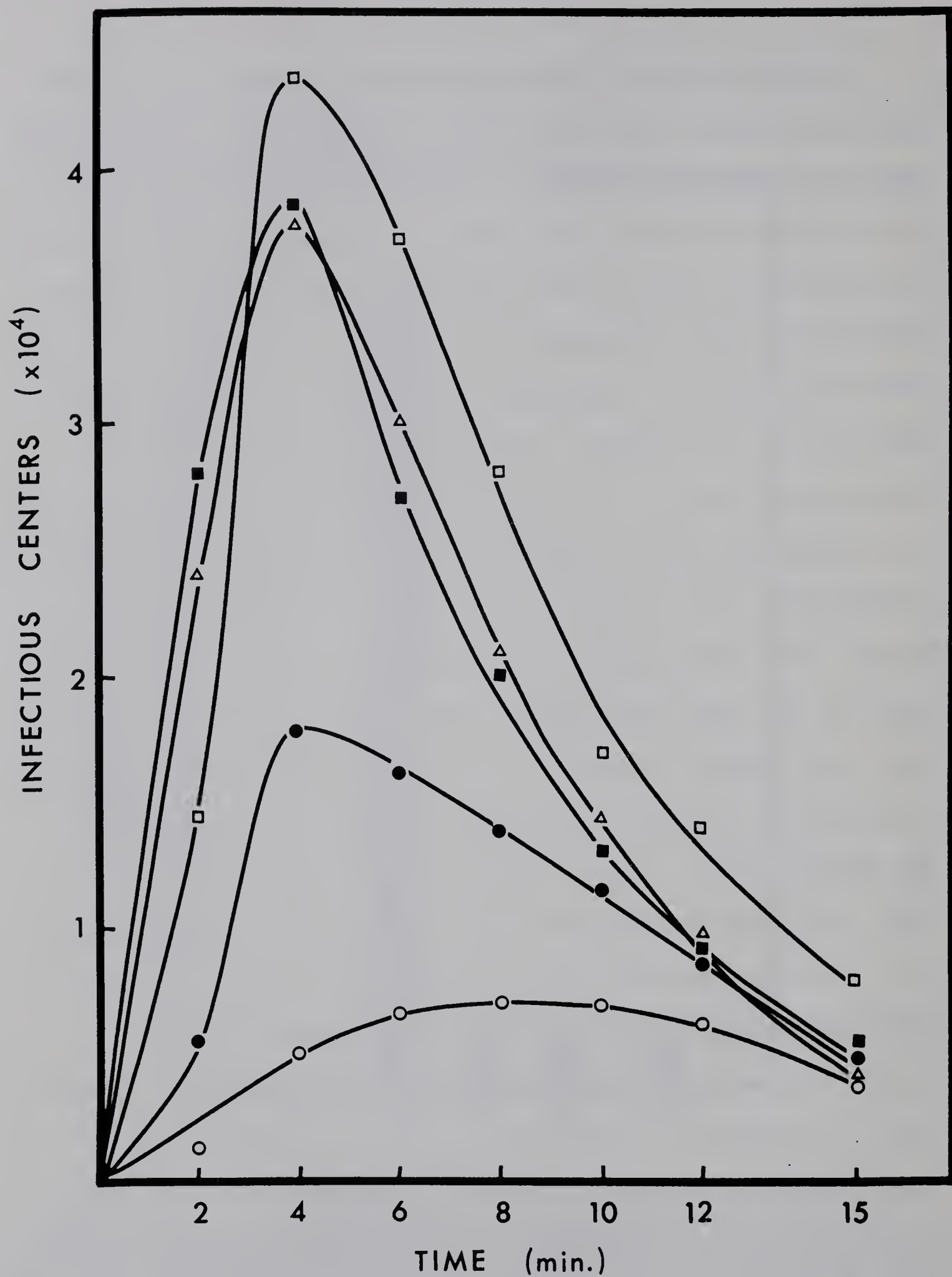


Figure 2.10. Infectious center formation in diploid cell-Mengo RNA mixtures incubated at 37° in (○) 0.6 M sucrose/PBS, (●) 0.8 M sucrose/PBS, (□) 1.0 M sucrose/PBS, (■) 1.2 M sucrose/PBS, (△) 1.4 M sucrose/PBS.

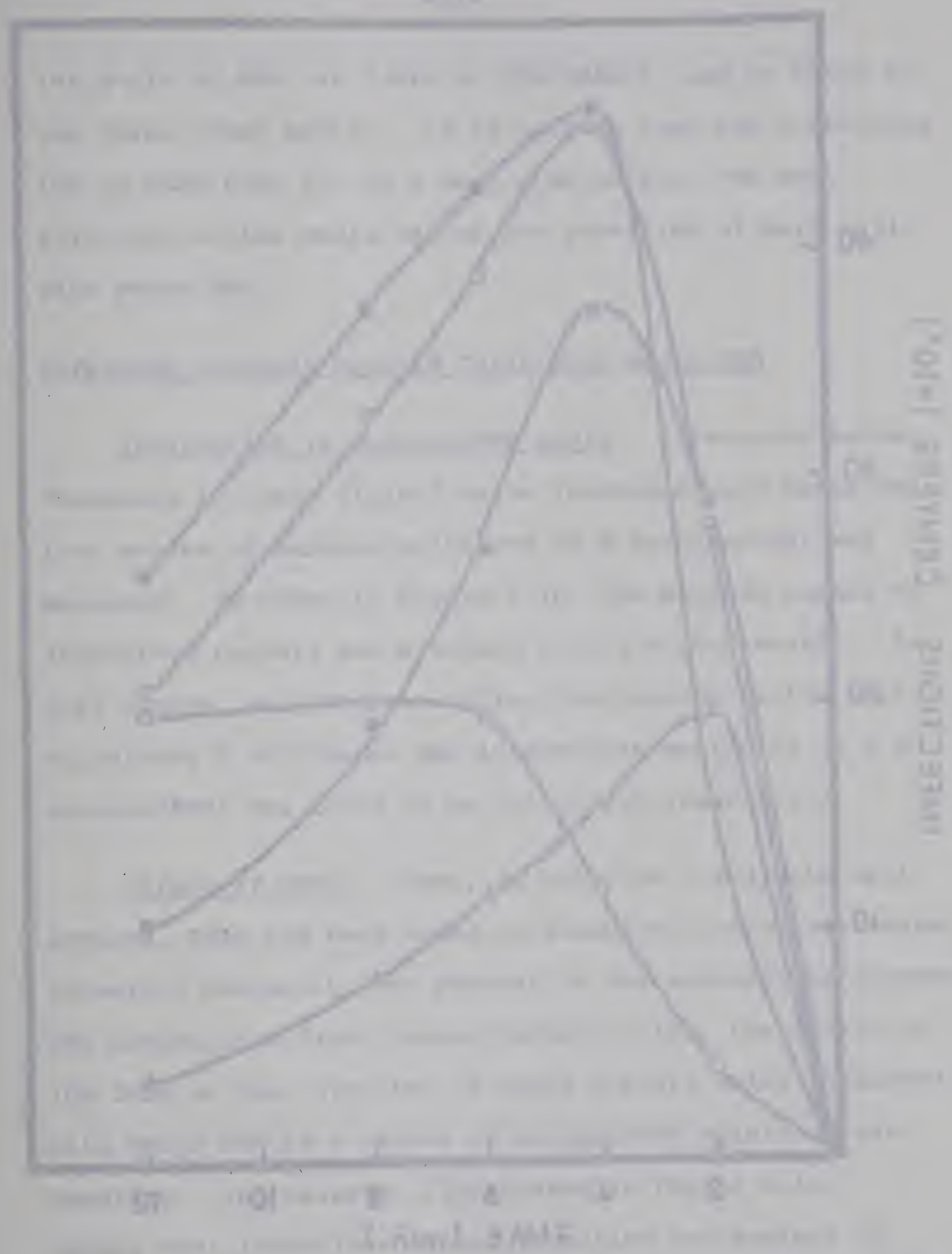


Fig. 1. Effect of pH on the activity of the enzyme. The substrate concentration was 0.1 M. The enzyme concentration was 0.01 M. The reaction was carried out at 25°C. The activity was measured by the amount of product formed in 10 minutes.

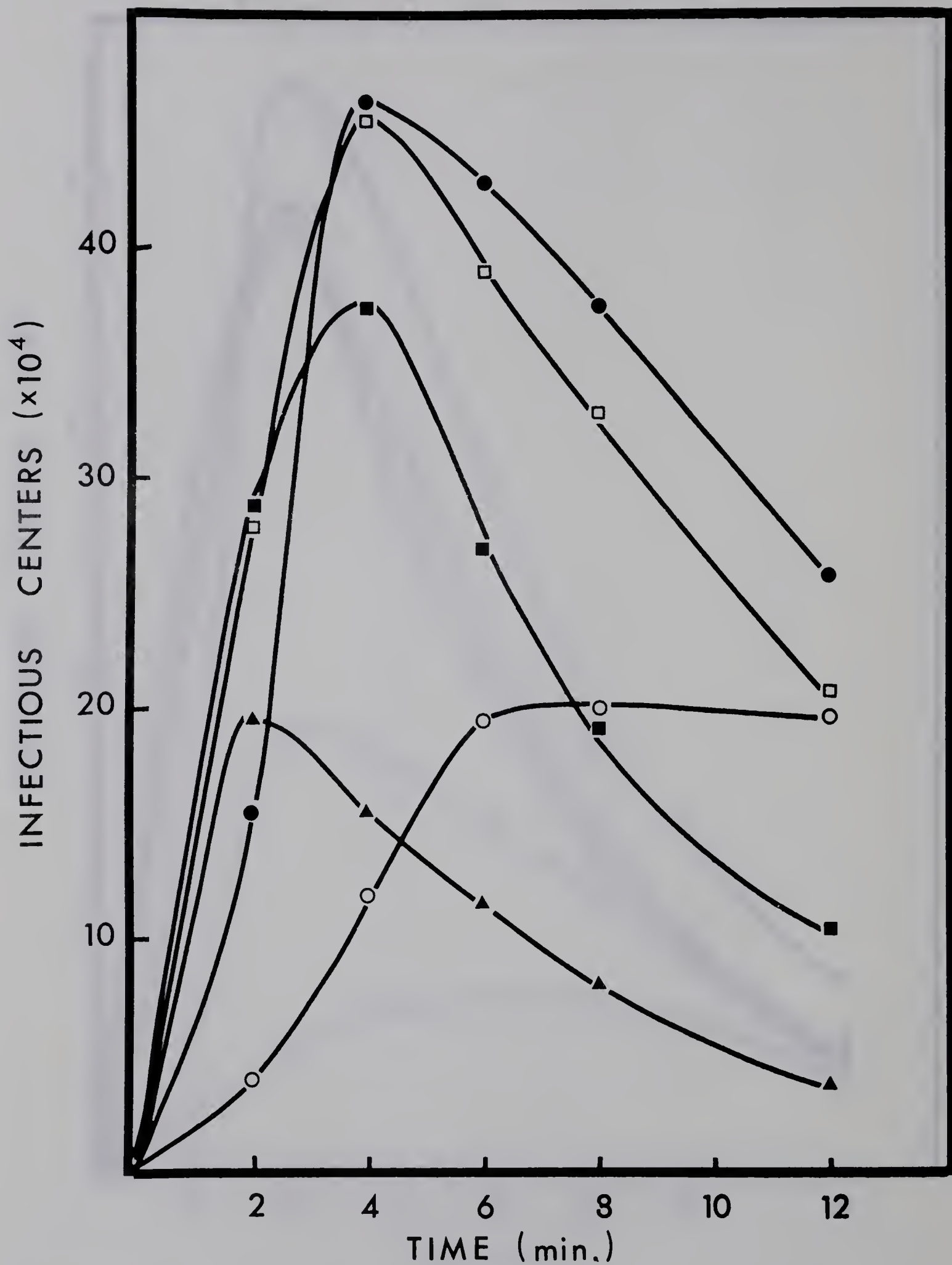


Figure 2.11. Infectious center formation in diploid cell-Mengo RNA mixtures incubated at 37° in (○) 0.4 M sucrose/PBS, (●) 0.6 M sucrose/PBS, (□) 0.8 M sucrose/PBS, (■) 1.0 M sucrose/PBS, (▲) 1.2 M sucrose/PBS. All media contained 10% DMSO.

(at 8 min in PBS, at 2 min in PBS-DEAE-D, and at 4 min in the three other media). It is obvious that PBS containing 100 μ g DEAE-D/ml is, by a very wide margin, the most efficient of the media tested for infection of HeLa cells with Mengo RNA.

Infection of Human Diploid Cells with Mengo RNA

Interaction in sucrose/PBS media. Infectious center formation in human diploid cells incubated with Mengo RNA in a series of sucrose solutions (x M sucrose/PBS) was measured. As shown in Figure 2.10, the maximum number of infectious centers was produced in 1.0 M sucrose/PBS. In this system, as with HeLa cells, the sucrose medium that stimulates L cell-Mengo RNA interaction maximally (0.6 M sucrose/PBS) was found to be relatively ineffective.

Effect of DMSO. Since, in both the L and HeLa cell systems, DMSO had been found to stimulate infectious center formation maximally when present in the appropriate sucrose/PBS medium at a final concentration of 10%, the effect of 10% DMSO on the infection of human diploid cells incubated with Mengo RNA in a series of sucrose/PBS solutions was examined. The results, illustrated in Figure 2.11, showed that infectious center formation was maximal in 0.6 - 0.8 M sucrose/PBS containing 10% DMSO. This points up a basic difference between the L cell system and those employing HeLa or human diploid cells. In the former, the

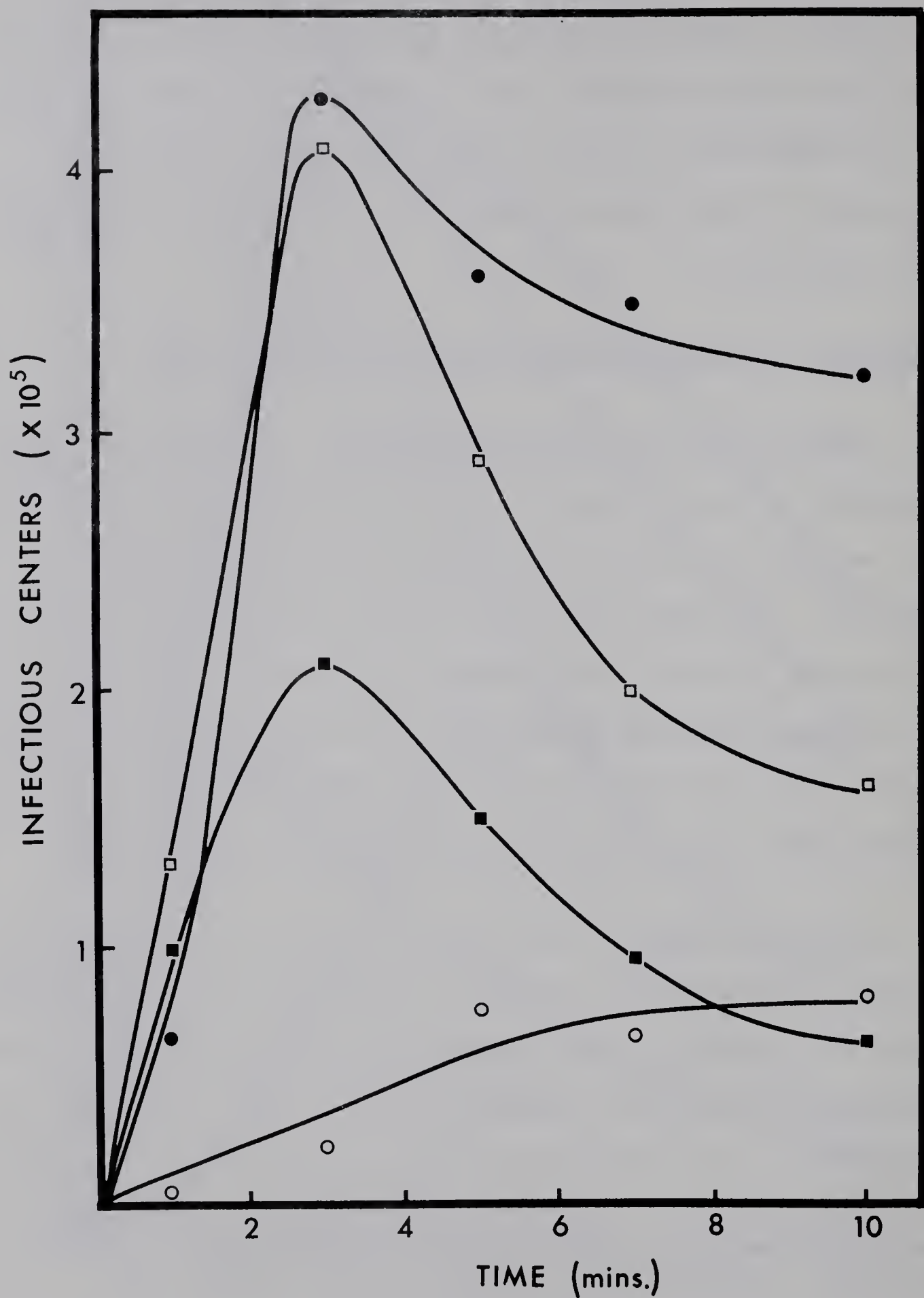


Figure 2.12. The effect of DMSO concentration on infectious center formation in diploid cell-Mengo RNA mixtures incubated at 37° in 0.8 M sucrose/PBS. (○) 5% DMSO, (●) 10% DMSO, (□) 15% DMSO, (■) 20% DMSO.

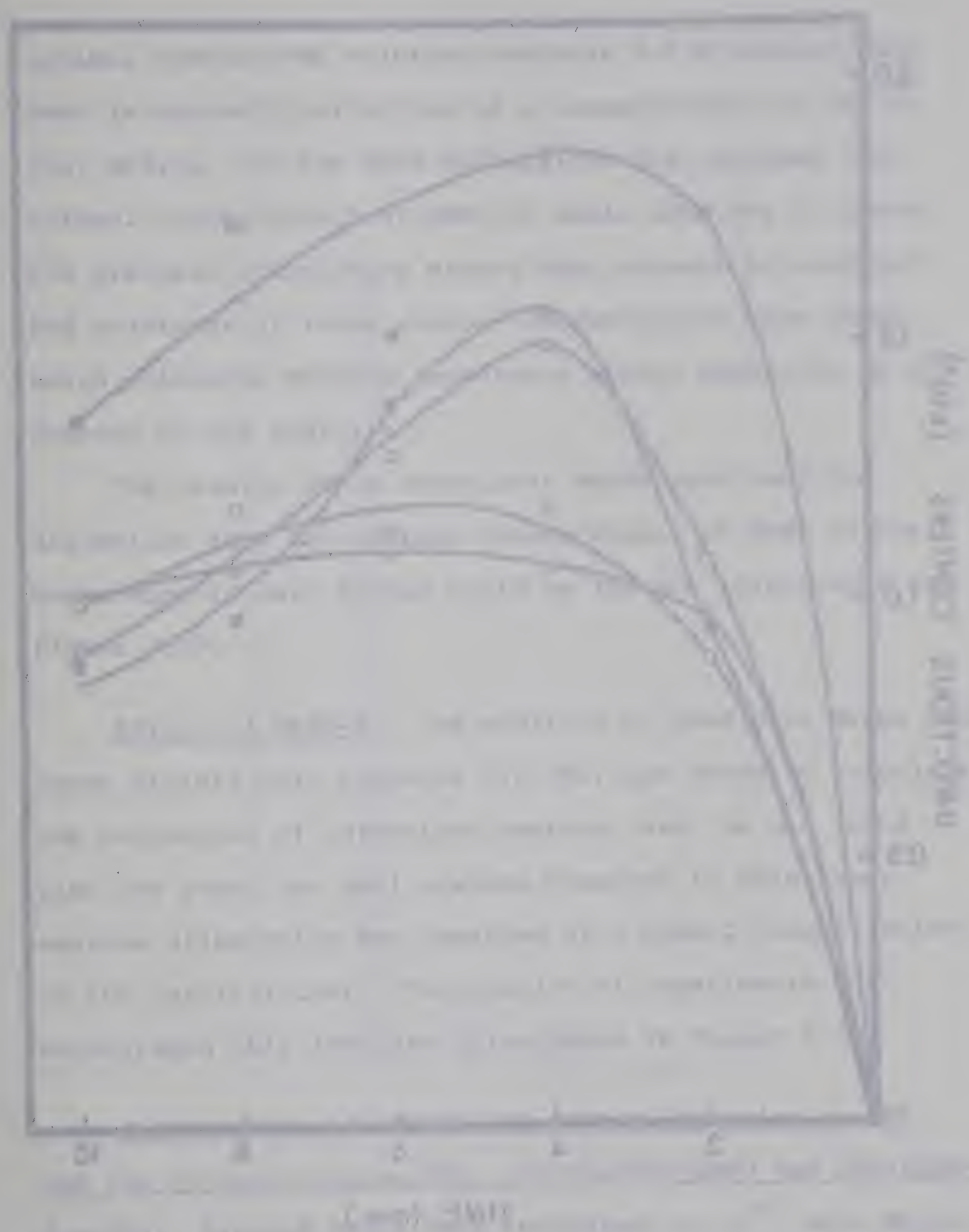


Figure 1. Effect of reagent concentration on the yield of the product. The curves show the percentage of product formed as a function of the reagent concentration for five different experimental conditions. The optimal reagent concentration for each condition is indicated by the peak of the curve.

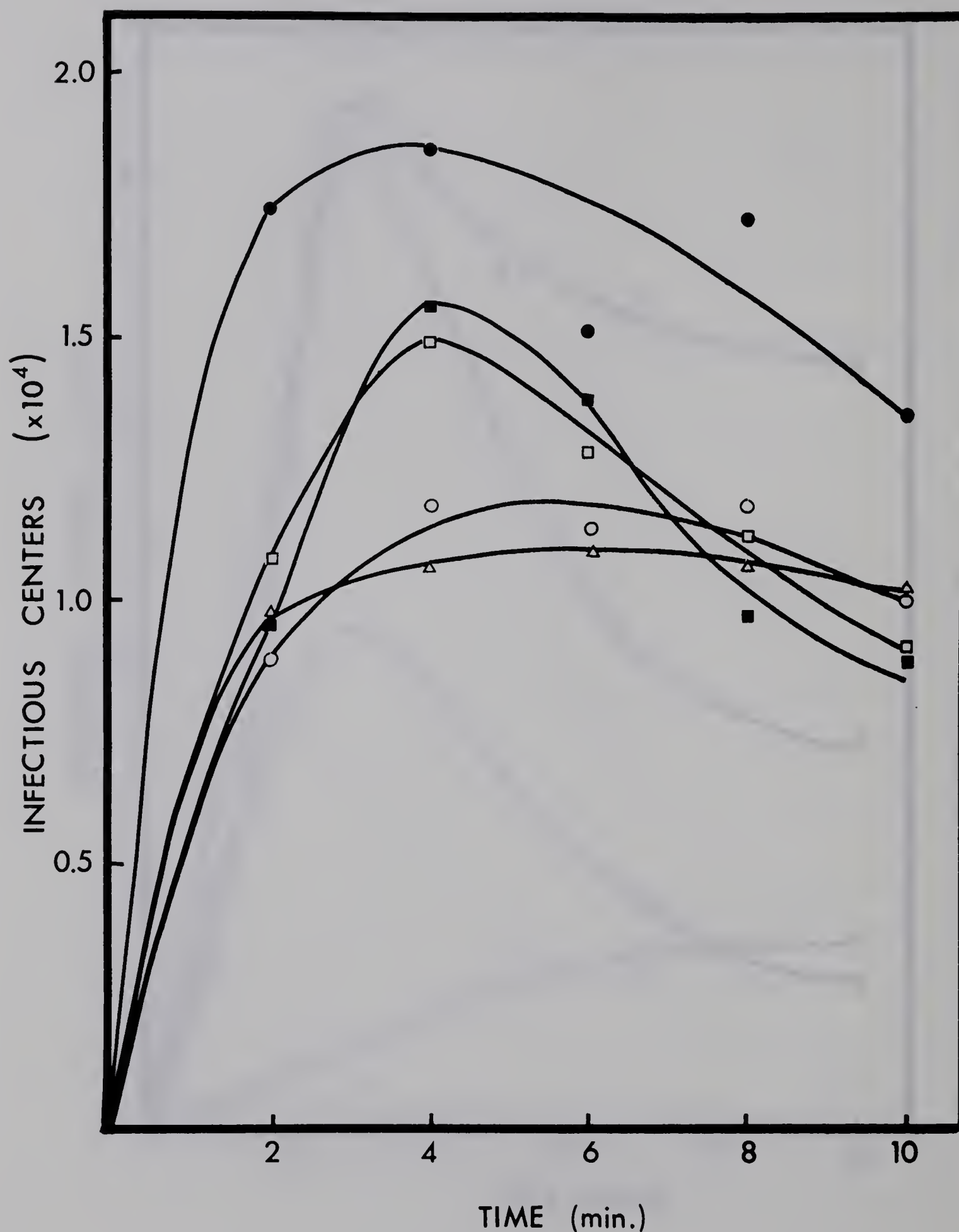


Figure 2.13. The effect of DEAE-D concentration on infectious center formation in diploid cell-Mengo RNA mixtures incubated at 37° in PBS. (○) 50 µg/ml, (●) 100 µg/ml, (□) 150 µg/ml, (■) 200 µg/ml, (Δ) 250 µg/ml.

optimal sucrose/PBS solution contains 0.6 M sucrose, and DMSO is optimally effective at a concentration of 10% in that medium. In the HeLa and diploid cell systems, the optimal concentration of DMSO is again 10%, but it exerts its greatest stimulatory effect when present in sucrose/PBS solutions of lower sucrose concentration than those which stimulate maximum infectious center formation in the absence of the additive.

The results of an experiment which confirmed the assumption that the optimal concentration of DMSO in the human diploid cell system would be 10% are illustrated in Figure 2.12.

Effect of DEAE-D. The addition of DEAE-D to Mengo RNA-human diploid cell mixtures (in PBS) was found to stimulate the production of infectious centers, and, as was found with the other two cell systems examined in this study, maximum stimulation was observed at a DEAE-D concentration of 100 μ g/milliliter. The results of experiments that established this fact are illustrated in Figure 2.13.

A comparison of infectious center formation in PBS and the optimal sucrose/PBS, sucrose/PBS-DMSO and PBS-DEAE-D media. Diploid cells were incubated (at 37°) with Mengo RNA in PBS, PBS containing 100 μ g DEAE-D/ml, 1.0 M sucrose/PBS and 0.6 M sucrose/PBS containing 10% DMSO, with each incubation mixture containing the same concentration of the

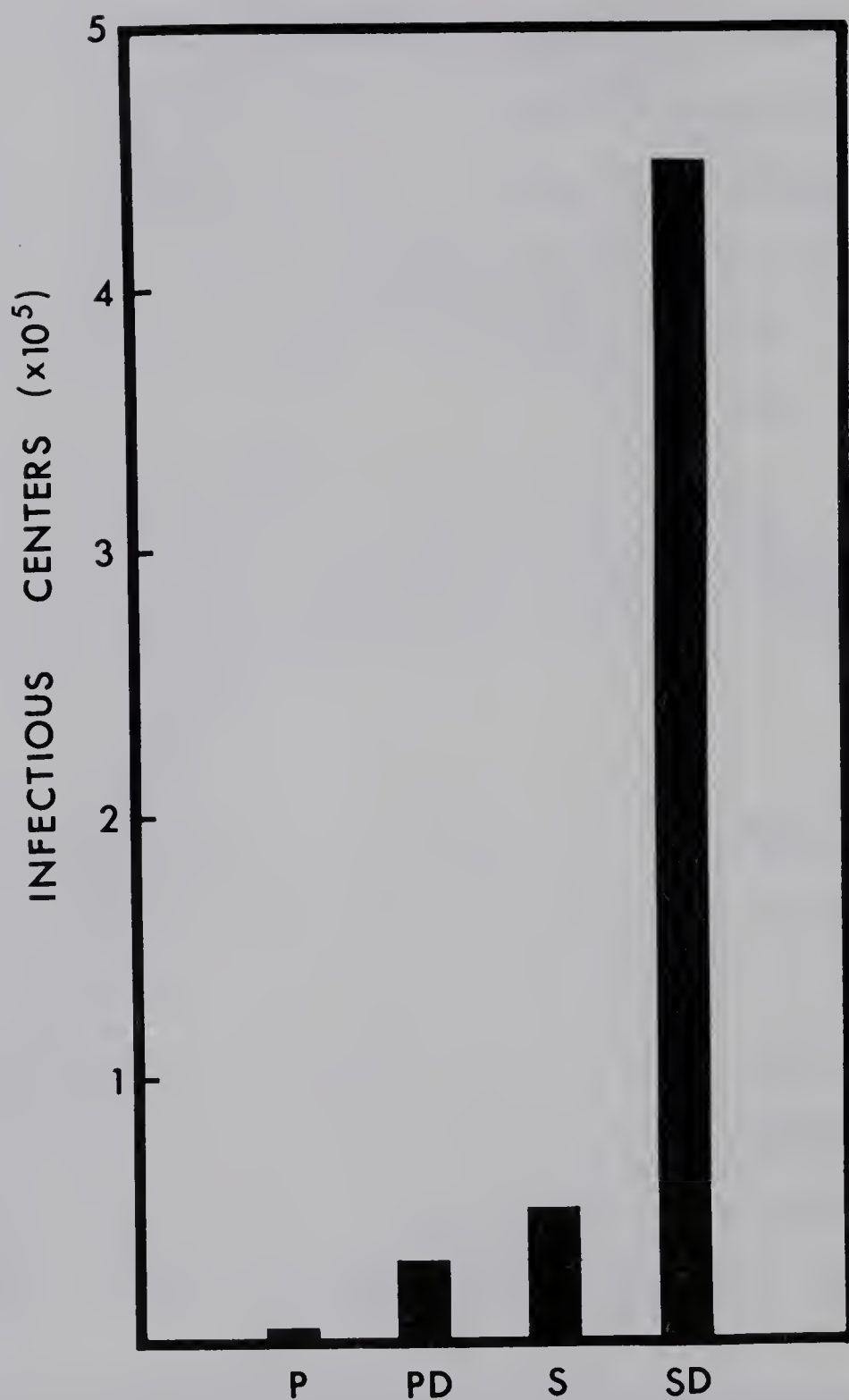


Figure 2.14. A comparison of infectious center formation in diploid cell-Mengo RNA mixtures incubated at 37° in (P) PBS, (PD) PBS containing 100 µg DEAE-D/ml, (S) 1.0 M sucrose/PBS, (SD) 0.6 M sucrose/PBS containing 10% DMSO.

same RNA preparation. Aliquots of each suspension were removed at intervals, and the number of infectious centers present in each was determined by titration on indicator monolayers of L cells. The results are illustrated in Figure 2.14. As was the case with the comparable studies with HeLa cells (see Figure 2.9) the values shown in this figure are the maximum number of infectious centers formed in each medium (it should be recalled that the time at which the maximum number of infectious centers is present varies from one incubation medium to another). Of the media examined in the Mengo RNA-diploid cell system, 0.6 M sucrose/PBS - 10% DMSO is, quite clearly, the one that stimulates most efficiently the productive interaction between cells and RNA. In this respect the diploid cells differ sharply from either L or HeLa cells, since with the latter two cell types, PBS containing 100 μ g DEAE-D/ml is the medium of choice. With diploid cells, fewer infectious centers are formed in PBS-DEAE-D than 1.0 M sucrose/PBS, and about 10 times as many infectious centers are produced in 0.6 M sucrose/PBS - 10% DMSO as in 1.0 M sucrose/PBS.

Comparison of Infectious Center Formation in L, HeLa and Diploid Cells

In order to make a direct comparison of the efficiency with which L, HeLa and diploid cells can be infected with Mengo RNA, infectious center formation was measured simultaneously in all three cell types in a number of incubation

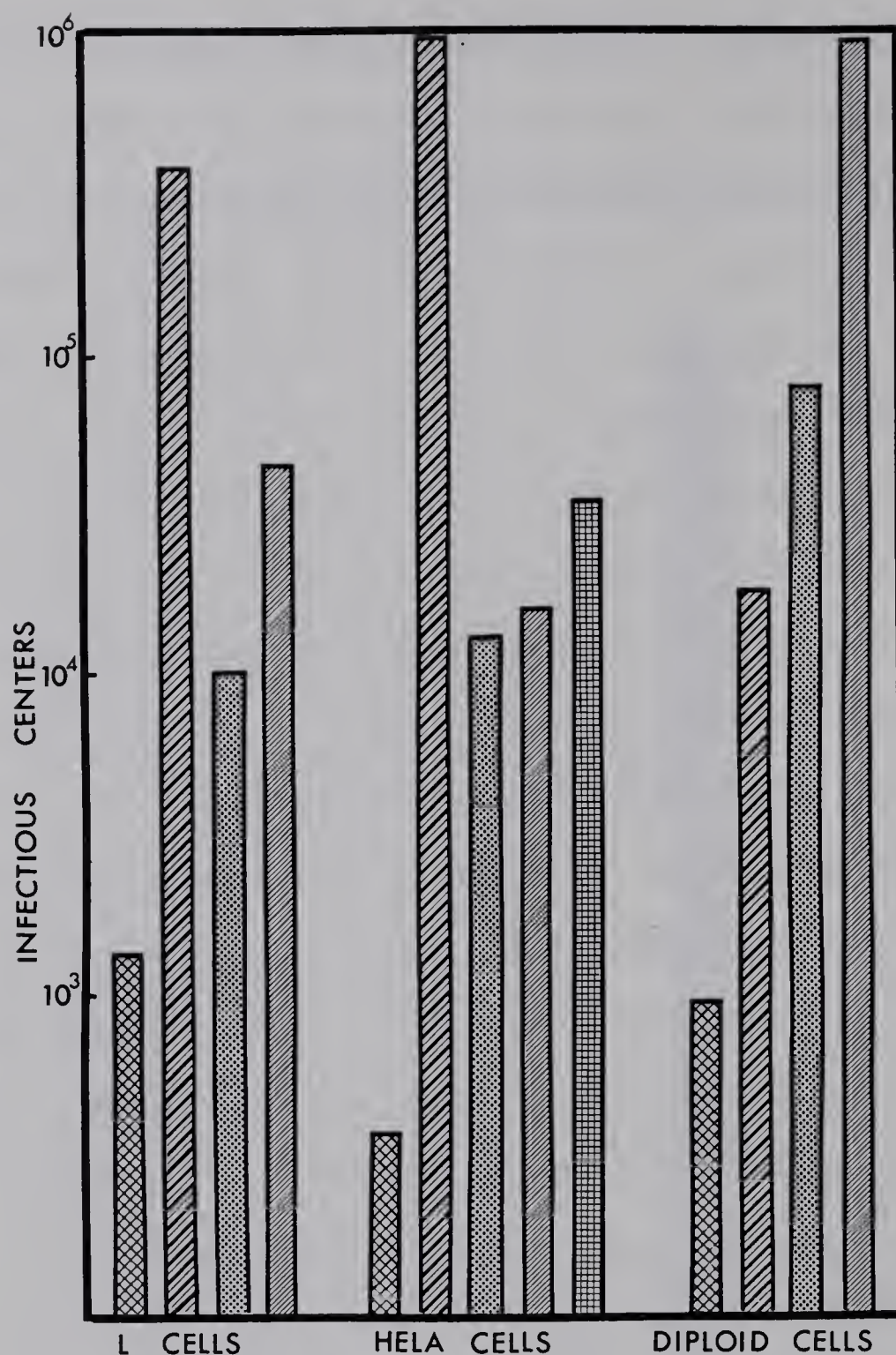


Figure 2.15. A comparison of infectious center formation in cell-RNA mixtures incubated at 37° in the following media with the given cell types:



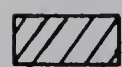
PBS (L, HeLa, diploid)



PBS containing 100 μ g DEAE-D/ml (L, HeLa, diploid)



0.6 M sucrose/PBS (L), 1.2 M sucrose/PBS (HeLa), 1.0 M sucrose/PBS (diploid)



0.6 M sucrose/PBS-10% DMSO (L, diploid), 0.8 M sucrose/PBS-10% DMSO (HeLa)



1.4 M PBS (HeLa)

media, using a single RNA preparation throughout. The media employed were PBS, PBS containing 100 μ g DEAE-D/ml, and the sucrose/PBS and sucrose/PBS - DMSO media that had been established as being optimal for each cell type. An additional medium - namely 1.4 M PBS - was included in the case of the HeLa cell system. All cell-RNA mixtures were incubated at 37⁰, aliquots were removed at intervals and titrated for infectious centers in the usual way. Data from three separate experiments were averaged and are shown in Figure 2.15. As was the case with the similar studies done separately with HeLa and diploid cells (see Figures 2.9 and 2.14), the values plotted here were taken from the peaks of the curves relating infectious center formation to time of incubation for each cell-incubation medium combination. Note, however, that unlike the other figures in this chapter, in Figure 2.15 the number of infectious centers formed are expressed on a logarithmic scale.

Several interesting differences in the productive interaction of Mengo RNA with cells of the three types may be noted from the data illustrated in Figure 2.15. HeLa cells were found to be less susceptible to infection with Mengo RNA in PBS alone than either L or diploid cells, whereas in the PBS-DEAE-D medium they, of the three cell types, were infected with the highest efficiency. In the HeLa cell system, DEAE-D was found to stimulate infectious center formation (relative to that observed in PBS) by a

factor of $>10^3$, while the stimulation produced in diploid cells was of the order of ten-fold. In contrast, DMSO was found to have its greatest enhancing effect with diploid cells, and to be only marginally effective in stimulating infectious center formation in HeLa cells. In the L cell system, the magnitude of the stimulation produced by each of DEAE-D and DMSO was found to be intermediate between that produced in the other two cell types.

Because the most notable differences in infectious center formation and the effects of DEAE-D and DMSO thereon were found between diploid cells on one hand, and the two established cell lines on the other, it was considered possible that the differences were due to differences in the ways in which the cells were cultivated and harvested rather than to basic differences among the cells. Both L and HeLa cells were maintained routinely in spinner cultures for at least 24 hours before they were used as assay cells in RNA titrations, whereas human diploid cells were grown on glass, and were removed therefrom by trypsin treatment only 2-3 hours prior to use. It was reasoned that trypsin might remove certain groups on the cell membrane necessary for the attachment of the DEAE-D-RNA complex. Were this the case, then the low degree of stimulation produced by DEAE-D in the diploid cell system, compared with that found in L and HeLa cells, could perhaps be explained on this basis. In an effort to resolve this question, L and HeLa

cells were treated with trypsin approximately one hour before being incubated with RNA in PBS-DEAE-D, sucrose/PBS, and sucrose/PBS-DMSO. Trypsin treatment neither decreased the efficiency with which the cells were infected in PBS-DEAE-D, nor increased the efficiency with which they were infected in sucrose-DMSO solutions. It was concluded, therefore, that the observed differences in the responses of the three cell types to the two additives reflect differences inherent in the cells themselves, and are not a consequence of variations in handling procedures.

Effect of Incubation Media on Cell Viability

It was considered possible that the differences among L, HeLa and human diploid cells with respect to infectious center formation in various incubation media might reflect differences in their resistance to the toxic effects of these media. For this reason, a limited study of the loss of cell viability in various media was undertaken. The experimental design was very simple. Aliquots of cells (L, HeLa or diploid) were suspended in PBS, 0.6 M sucrose/PBS, 0.6 M sucrose/PBS - 10% DMSO, PBS containing 100 μ g DEAE-D/ml, and PBS containing 20 μ g polyornithine (mol. wt. 150,000)/milliliter. After incubation for 10 min at 37^o, the suspensions were diluted with the appropriate growth medium, a sample was removed from each and the per cent of cells capable of absorbing neutral red (i.e. viable cells) was measured. The remainder of each suspension was added to

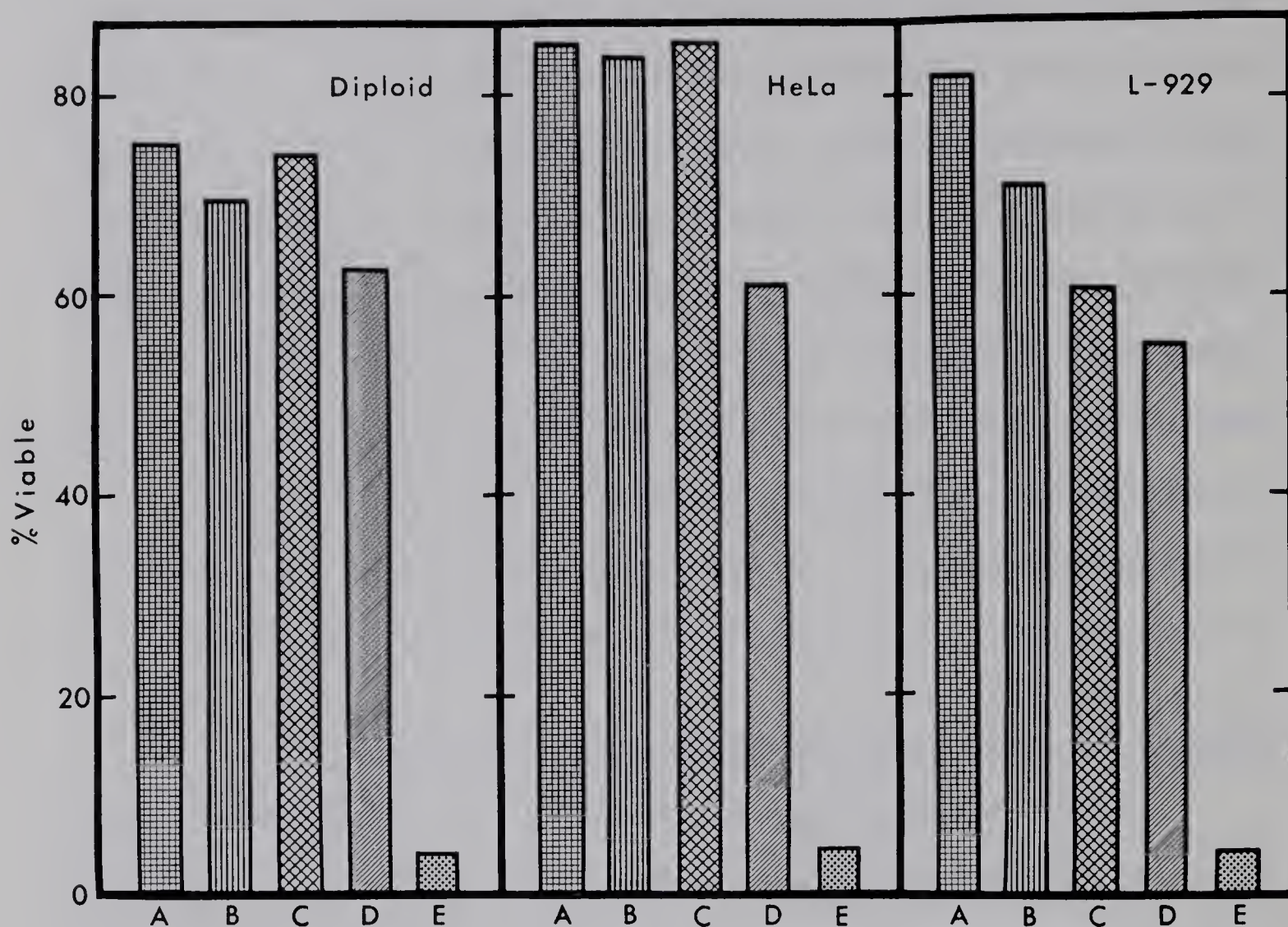


Figure 2.16. Viability of cultured cells, as determined by neutral red staining, following a 10 min incubation at 37° in:

- A - PBS
- B - 0.6 M sucrose/PBS
- C - 0.6 M sucrose/PBS-10% DMSO
- D - PBS containing 100 µg DEAE-D/ml
- E - PBS containing 20 µg polyornithine/ml

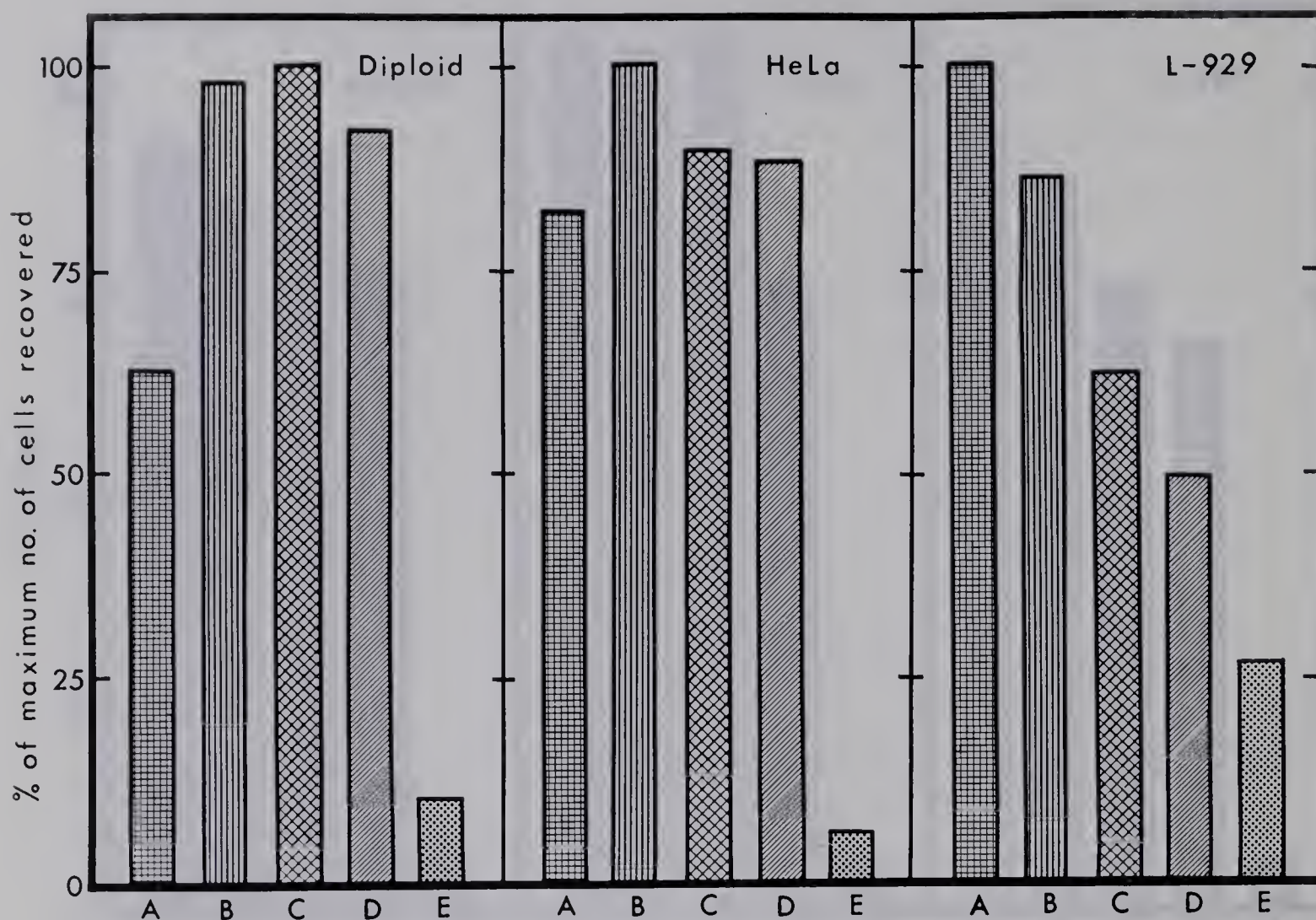


Figure 2.17. Viability of cultured cells, as determined by their ability to attach and/or grow, following a 10 min incubation at 37° in:

- A - PBS
- B - 0.6 M sucrose/PBS
- C - 0.6 M sucrose/PBS-10% DMSO
- D - PBS containing 100 µg DEAE-D/ml
- E - PBS containing 20 µg polyornithine/ml

After incubation, the cells were centrifuged, washed with PBS, resuspended in growth medium and inoculated into duplicate plates. After 24 hrs at 37° the plates were washed 2x with PBS, and the cells removed by scraping (rubber policeman) and counted.

For comparative purposes between the 3 cell lines, viability is expressed as a % of the maximum number of cells recovered, which, with all 3 cell types, was at least half of the number introduced into the petri dishes.

a plastic petri dish, incubated overnight at 37⁰, after which cells which had become attached to the plastic surface (i.e. viable cells) were removed and counted. The results of these experiments are summarized in Figures 2.16 and 2.17. A comparison of the two indicates that estimates of cell viabilities by these two methods gave very similar results. HeLa and human diploid cells appear to be somewhat more resistant to loss of viability in sucrose solutions of elevated osmolarity than are L cells, which may explain why the optimal sucrose/PBS solutions for HeLa and diploid cells are of a higher sucrose concentration than the solution that is optimal for L cells. In the studies described in Chapter 1, in which the ability of cells to support viral replication was used as the criteria of viability, DMSO appeared to protect L cells from loss of viability when incubated in 0.6 M sucrose/PBS (see Figure 1.6), but this earlier observation was not borne out by these later studies. From the point of view of the overall study, perhaps the only conclusion that may be drawn from these experiments is a negative one,- namely that there is (with the exception of the highly toxic polyornithine) no obvious correlation between the ability of cells to survive in a particular medium and the efficiency with which they can be infected therein.

Subsequent to these studies, polyornithine of molecular weight 21,000 was obtained and was found to produce a slight stimulation in infectious center formation when

added to L cells incubated with Mengo RNA in PBS. Its effect on the viability of the three cell types was measured by neutral red staining. It was found that after a 10 min incubation at 37° in PBS containing 20 µg "low molecular weight" polyornithine/ml, 60%, 70% and 50% of L, HeLa and diploid cells, respectively, retained their viability.

Discussion

The studies summarized in this chapter, in which the optimal conditions for the interaction of Mengo RNA with HeLa and human diploid cells were defined, and in which the efficiencies with which L, HeLa, and human diploid cells were infected with Mengo RNA in a variety of media were compared, make it clear that the optimal conditions for the formation of infectious centers in viral RNA-cell systems depend upon the cell type used.

That this is so has been demonstrated, at least when the data are viewed in retrospect, by the work of other investigators. For example, Holland et al. (1959a) showed that fewer plaques were produced by poliovirus RNA in monolayers of primary human amnion cells than in either HeLa or monkey kidney (MK) monolayers (when all three cell types were infected under the same conditions). The same investigators reported that certain cell types (human amnion, cottontail rabbit papilloma, cottontail rabbit epithelium) produced low yields of virus, while others (domestic rabbit

fibroblasts, HeLa, and rabbit skin cells in primary culture) produced much higher yields of virus after infection with polio RNA (Holland et al., 1959b). Again, all cell types were infected under the same conditions, and it is not possible to determine from the data whether the low virus-yielding cells were infected with lower efficiency than were cells in the other group or whether they produced fewer progeny virus particles per infected cell than did the other cell types. The first of the two alternatives seems the most likely in the light of subsequent studies by Holland et al. (1960b), in which they found that the number of virus particles produced per polio RNA-infected cell was approximately the same for L and HeLa cells.

The first extensive study of the kind described in this chapter was that reported by Igarashi et al. (1963) who employed a suspended cell system for the assay of RNA isolated from Japanese encephalitis virus. Cells of three established lines (HeLa S3, KB, and MS) which could not be infected by the intact virus, as well as susceptible chick cells, were incubated with RNA dissolved in solutions containing various concentrations of NaCl in 0.02 M phosphate buffer, pH 7.4, and titrated for infectious centers on monolayers of chick cells. The optimal conditions (concentration of NaCl and time of incubation at 37°) for infectious center formation were found to be different for each cell type.

RNA isolated from Mengo virus (M variant) was used in

all the work reported in this thesis, so no unequivocal conclusions can be drawn concerning the importance (if any) of the particular RNA in determining the conditions for its optimal interaction with cultured cells. However, the data presently available suggest that the nature of the RNA is less important in this regard than is cell type.

In an earlier study, Ellem and Colter (1961a) compared infectious center formation in the HeLa cell-polio RNA and L cell-Mengo RNA systems, employing the two series of solutions, x M PBS and x M sucrose/PBS. They reported that in these two series of incubation media, maximum numbers of infectious centers were produced in HeLa cell-polio RNA mixtures when incubations were carried out in 0.9 M PBS and in 0.98 M sucrose/PBS, and that many more were formed in hypertonic saline than in the sucrose media. In the present study, it was found that maximum numbers of infectious centers were produced in HeLa cell-Mengo RNA mixtures at somewhat higher NaCl and sucrose concentrations than those reported by Ellem and Colter. However, the general pattern was the same, in that the hypertonic saline solution was found to promote infectious center formation more effectively than did the sucrose medium, and that the concentration of sucrose which gave optimal interaction between HeLa cells and Mengo RNA was considerably higher than that which was optimal for L cell-Mengo RNA interaction. Rouhandeh (1965) assayed RNA isolated from three different strains of

poliovirus, and from Coxsackie A7, and ECHO 8 viruses, in monolayers of MK or HeLa cells and in four different diluents (1 M NaCl, 2 M MgSO_4 , 0.05% $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ in physiological saline, and physiological saline containing 400 μg histone/ml). He found that with each cell type, the relative efficiencies with which plaques were produced in the four diluents were the same for all five species of RNA.

The results discussed in the preceding paragraph are suggestive, but far from conclusive. Since all the RNA preparations used in the studies cited above were isolated from picornaviruses, they might be expected to be structurally and biologically similar. It would be of interest (and necessary before firm conclusions could be drawn) to assay RNA, isolated from unrelated viruses, in cells of one or more types.

At present, it is not possible to define precisely those differences among HeLa, L, and diploid cells that are responsible for the observed differences among them with respect to their interaction with Mengo RNA in the various incubation media examined in this study. There does not appear to be any relationship between loss of cell viability in these media and the efficiency with which cells are infected therein, and the basic difference between diploid cells and the two established lines does not appear to be explicable on the grounds that they are handled differently prior to use. One is left with the

proposition that the observed differences reflect differences in certain intrinsic properties of the cells, and in this respect, it is interesting to compare the results obtained by Amstey and Parkman (1966) with those described in this chapter. These investigators reported that, in primary cultures of African green monkey cells, they obtained a ten-fold higher titer of polio RNA in the presence of DMSO than was produced by the addition of DEAE-D, a finding which is similar to the one described here concerning the effects of the two additives on infectious center formation in the human diploid cell-Mengo RNA system. It is interesting, although certainly premature, to speculate that DMSO is more effective than DEAE-D in stimulating the infectivity of viral RNA in diploid (normal) cells, and that DEAE-D is the more effective additive in cells (aneuploid) of established lines.

Whatever the basis may be for the differences among various cell types with respect to the conditions that are optimal for cell-viral RNA interaction, the data presented in this chapter leave no doubt that very real differences do exist. They underscore the hazards of extrapolating the results obtained from studies of one cell-RNA system to studies of a different cell-RNA system. In fact, considering the differences between the observations described here and those published earlier by Ellem and Colter (1961a) concerning optimal conditions for HeLa cell-viral RNA

interaction, it would appear that such extrapolations cannot be made with confidence even when the same cell type is used in separate laboratories. If there is a lesson to be learned from these studies, it is that for any investigator to carry out meaningful studies of cell-viral RNA interaction, he must first define carefully the optimal conditions for the particular cell type(s) with which he is concerned.

CHAPTER 3

Investigations of the Mechanisms by which Sucrose, DMSO, and DEAE-D Stimulate Cell-RNA Interaction

Introduction

The stimulatory effects of sucrose, DMSO, and DEAE-D on the formation of infectious centers in cultured cell-Mengo RNA mixtures have been described in Chapters 1 and 2. The results summarized therein provide answers to questions concerning the concentrations of these substances and the conditions of incubation that lead to maximal cell-RNA interaction, but do not provide any insight into the more interesting questions concerning the mechanisms by which these substances exert their stimulatory effects.

The results of early studies of the infection of cultured cells by viral RNA in solutions of elevated osmolarity led certain investigators to suggest that the stimulation in infectivity observed in such systems was the result of an inhibition of extracellular and/or intracellular ribonucleases. DMSO has been shown to increase the permeability of cell membranes to numerous, unrelated molecules, which suggested that the presence of DMSO in the medium in which cells and RNA are incubated might increase the amount of RNA taken up by the cells. The chemical nature of the DEAE-D molecule suggested that binding between molecules of the viral RNA and the

polycation could be a factor in the stimulation of infectious center formation by this compound. The results of investigations designed to consider these and other possible mechanisms by which sucrose, DMSO and DEAE-D stimulate infectious center formation by Mengo RNA are summarized in this chapter. Although the data collected have not made it possible to define precisely the mechanisms of action of these compounds, they do provide considerable information concerning some of the factors involved in cell-RNA interaction in the media examined in this study.

Materials and Methods

Purification of M-Mengo Virus

Purified virus was obtained by the method described previously by Scraba et al. (1967). It is outlined below.

Suspensions of methanol-insoluble material from Povitsky or roller bottles (see Routine Materials and Methods section for details concerning the propagation and concentration of the virus) were subjected to sonic oscillation for 60 sec at maximum output in a Raytheon Model DF 101 oscillator (Raytheon Company, Waltham, Mass.) in order to disrupt large aggregates. A solution of α -chymotrypsin (3 times crystallized, Worthington Biochemicals, Freehold, N.J.) was added to give a final enzyme concentration of 0.8 mg/ml, and the mixture was incubated at 37° for 45 minutes. An equal volume of 0.2 M sodium pyrophosphate (pH 8.0) was then added, followed by ribonuclease

(crystalline, salt-free; Worthington Biochemicals) to give a final concentration of 0.08 mg/ml, and incubation was continued for 30 minutes. The mixture was then chilled and clarified by centrifugation at 8000 g for 30 minutes. Virus was recovered from the supernatant by centrifugation at 100,000 g for 60 min, and was resuspended in a few ml of 0.02 M potassium phosphate buffer, pH 7.1.

Final purification of the virus involved chromatography of the enzyme-treated suspension on a column of hydroxylapatite, equilibrated and run at 4°. The adsorbent was prepared by the method of Levin (1962), except that one-fourth or one-third the recommended quantities of ingredients were used. A column of dimensions 1.6 x 22 cm (diameter x length) was packed under pressure and washed with about 300 ml of 0.13 M potassium phosphate buffer (pH 7.1). The virus suspension was added to the packed, equilibrated column, and allowed to soak in under gravity. Two small volumes of 0.13 M buffer were added to wash in the virus sample. Material was eluted from the column by means of a concave phosphate buffer gradient, produced by using 3 chambers in a Varigrad mixing device (Buchler Instruments Inc., Fort Lee, N.J.). The starting phosphate concentration was 0.13 M, and the limiting concentration was 0.8 M. A flow rate of 0.5 ml/min was maintained by a peristaltic pump, and 40 5-ml fractions were collected. The absorbance (at 260 m μ) of each fraction was measured in a Beckman DU spectrophotometer, and the fractions that

comprised the virus peak were pooled for extraction of the RNA.

Isolation of RNA from Purified Virus

RNA was isolated from freshly purified virus by the modified sodium dodecyl sulfate-phenol procedure described by Scraba et al. (1967) in which Macaloid (Stanley and Bock, 1965) and dextran sulfate (Dickman, 1958) were added to inhibit any ribonucleases which may have been present. All glassware coming into contact with the RNA solution was sterilized beforehand; and all manipulations were carried out between 0 and 5° unless otherwise indicated.

The pooled fractions from the column chromatography step were concentrated in a collodian bag apparatus (Carl Schleicher and Schuell Co., Keene, N.H.) to a volume of approximately 10 milliliters. This suspension was then made 0.001 and 0.002 M with respect to EDTA and $MgCl_2$ respectively, and sodium dodecyl sulfate (SDS) and dextran sulfate (molecular weight 60,000 to 90,000; Sigma Chemical Co., St. Louis, Mo.) were added to give final concentrations of 1% and 0.005% respectively. After incubation at 37° for 10 min and subsequent chilling in ice, a Macaloid suspension was added to give a final Macaloid concentration of 0.2%. Protein was removed by shaking for 1 min with an equal volume of water-saturated phenol. The resulting emulsion was centrifuged at 2000 g for 10 min, and the upper aqueous layer was removed without disturbing the

interface. Macaloid and water-saturated phenol were added to this aqueous solution and the extraction procedure was repeated twice. Residual phenol was removed by extracting the final aqueous solution 6 times with equal volumes of ether, and the ether remaining after this step was removed by bubbling nitrogen through the solution for 30 minutes. The RNA solution was stored at -60° .

Preparation of ^3H -labelled M-Mengo RNA

Pools of ^3H -labelled virus were prepared as described in the Routine Materials and Methods section, except that for a period of 2 hours prior to infection, the cells were maintained in growth medium containing 1% horse serum and actinomycin D (a gift of Merck, Sharp and Dohme Research Laboratories, Rahway, N.J.) at a concentration of $2\text{ }\mu\text{g}/\text{milliliter}$. During the period of viral growth, this medium was supplemented by the addition of ^3H -uridine at a level of $4\text{ }\mu\text{C}/\text{milliliter}$. The procedures described above were used to purify the labelled virus and to isolate the RNA therefrom.

Measurement of radiolabel

Both sucrose and DEAE-D were found to decrease the efficiency with which ^3H -RNA could be counted, and it was necessary to employ one of two different methods to eliminate the quenching observed in the presence of these compounds.

(a) If high levels of radioactivity were present, samples containing sucrose (0.6 M in the case of incubation media, or 5-20% in fractions collected from sucrose gradients) were simply diluted 1:10 in deionized water and either 0.1 or 0.2 ml aliquots of the diluted samples were added to 10 ml of p-dioxane-based scintillation fluid for counting. It was established that such a dilution reduced the sucrose concentration to a level below that which introduced errors due to quenching.

(b) Sucrose-containing samples which contained a limited amount of radioactivity, or those in which DEAE-D was present were collected or pipetted onto Whatman filter paper discs (3 cm diameter) which were then immersed in cold (4°) 10% trichloroacetic acid (TCA). After 60 min, the filters were removed and washed with four successive portions of cold 5% TCA, the filters being suspended in each wash medium for at least 10 minutes. This was followed by a wash with a 1:1 mixture of ethanol-diethyl ether (at 35°), and finally a wash in diethyl ether alone. The filters were then air-dried and suspended in 5 ml toluene-based scintillation fluid for radioactivity measurement.

Scintillation fluid. In the early stages of this work, the radioactivity in aqueous samples was measured in 10 ml of the dioxane-based scintillation fluid described by Bray (1960). Later, a simpler mixture recommended for use in Beckman liquid scintillation counters was tested and

found to be as satisfactory as that described by Bray. This mixture, used in all of the subsequent experiments, was prepared by dissolving 100 gm naphthalene and 5 gm 2,5-diphenyloxazole (PPO) in 1 liter of p-dioxane.

Radioactivity on filter paper discs was measured by suspending the filters in 5 ml of scintillation fluid of the following composition: 6.0 gm PPO and 0.5 gm 1,4-bis[2-(5-phenyloxazolyl)] benzene (POPOP) per liter of toluene.

Measurement of Uptake of ^3H -RNA

L cells (harvested from spinner cultures and washed twice in PBS containing 0.05% BPA) were incubated, at a concentration of 5×10^6 cells/ml, with ^3H -Mengo RNA in each of the media that were examined. The suspensions were incubated in a water bath at 37° . At intervals, 1 ml aliquots were removed and added to 9 ml volumes of growth medium. The cells were sedimented by centrifugation, washed once in 5 ml of growth medium, resuspended in a small volume of deionized water (usually 0.6 ml) and disintegrated by sonic vibration (5 min at maximum output in a Raytheon Model DF 101 oscillator). Aliquots of each cell lysate were added to vials containing 10 ml p-dioxane-based scintillation fluid, and the radioactivity in each was measured in a Beckman (model CPM-100) liquid scintillation spectrometer.

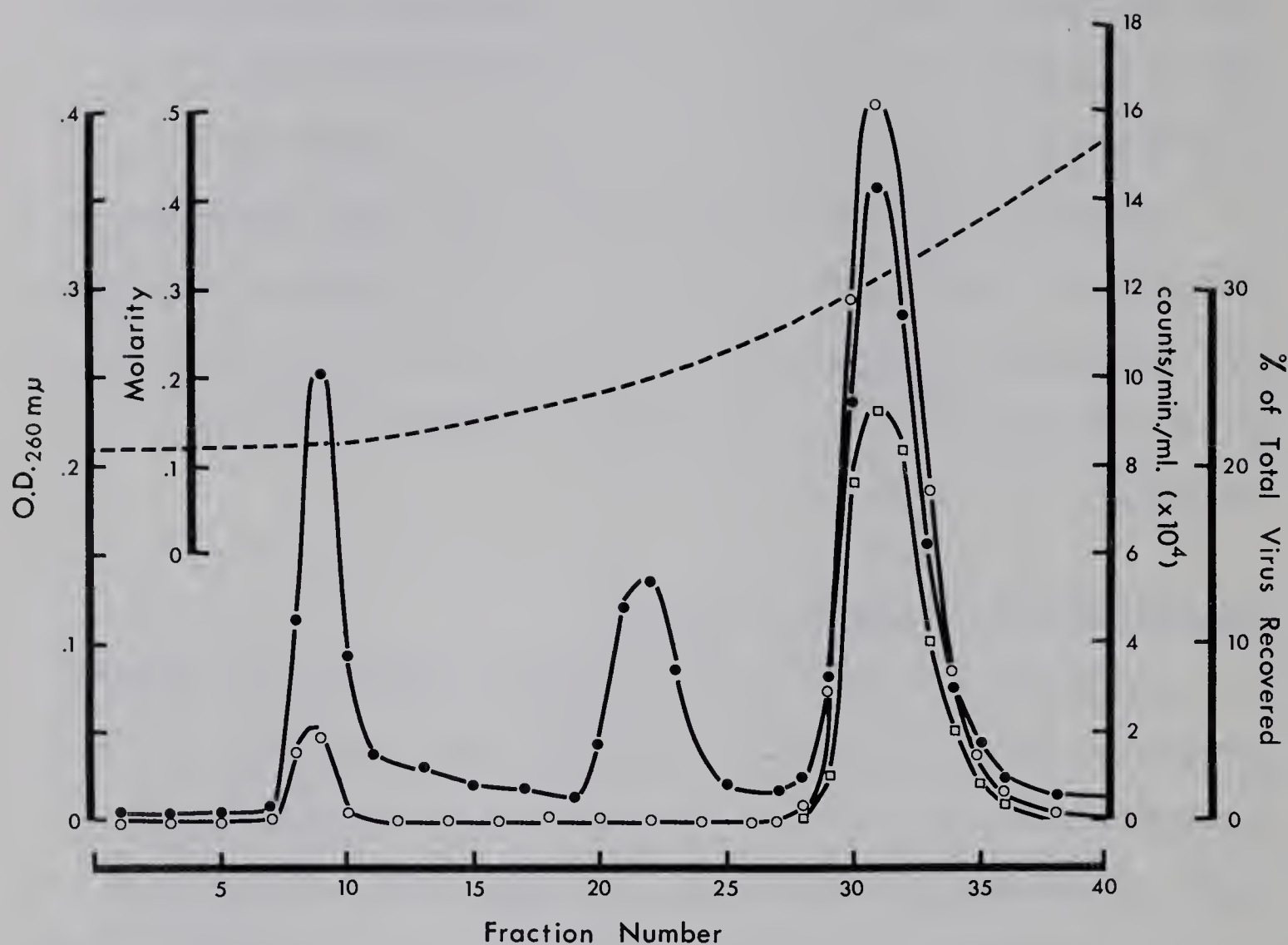


Figure 3.1. Column chromatography on hydroxylapatite of partially purified ³H-labelled Mengo virus. The pH of the eluting potassium phosphate buffer was 7.1. Conductivities of each fraction were measured on a conductivity meter, type CDM-2d (Radiometer, Copenhagen, Denmark), and these values were converted to phosphate molarities using a standard curve.

- = absorbance profile (260 mμ)
- = radioactivity profile
- = infectious virus, measured by plaque titration

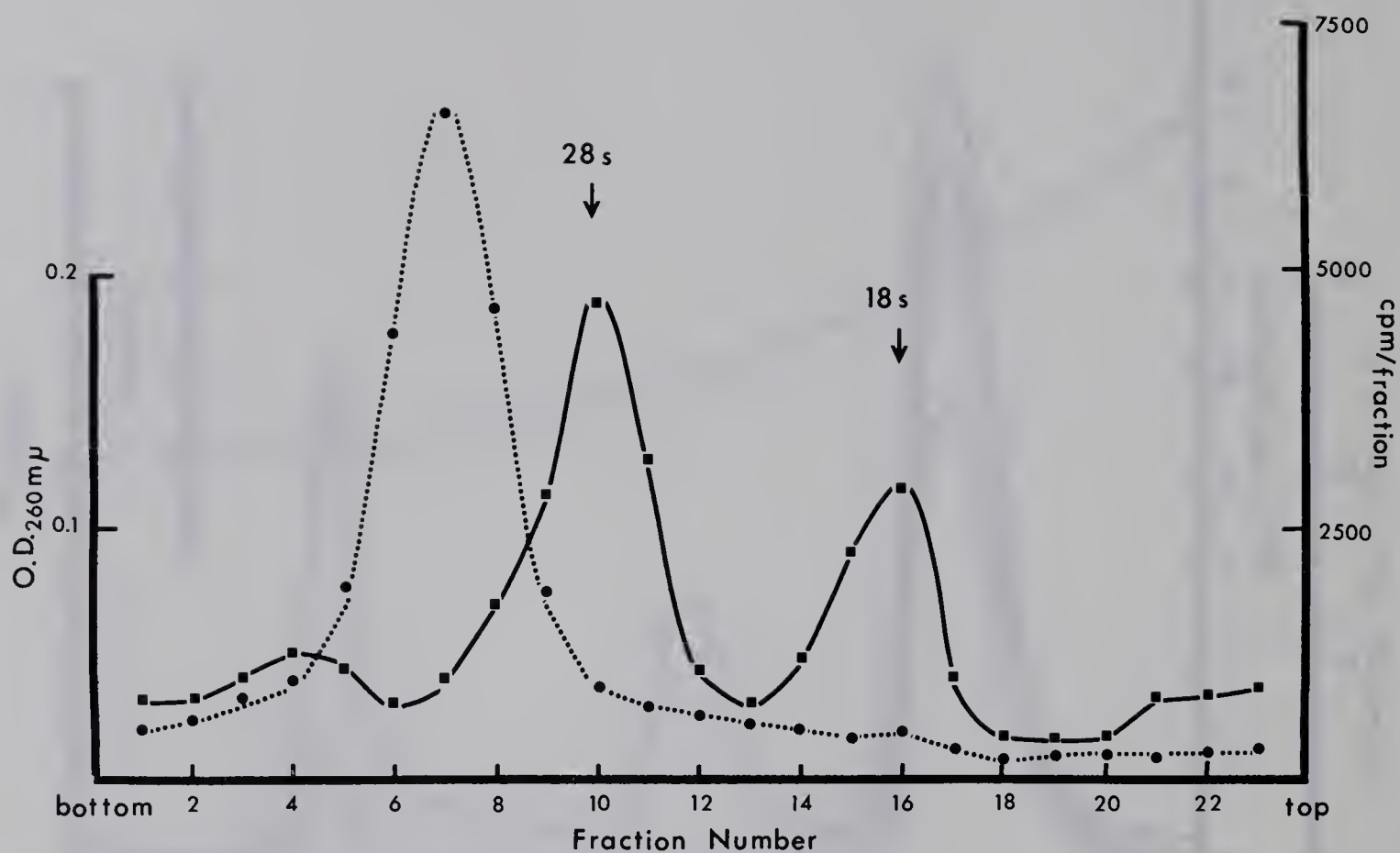


Figure 3.2. Sucrose density gradient (5-20%) sedimentation analysis of ^3H -labelled RNA isolated from highly purified Mengo virus, and containing ribosomal RNA (from L cells) as an O.D. marker. Centrifugation was for 3 hr at 35,000 rpm in a Spinco SW39 swinging bucket rotor. (—) = O.D. $_{260\text{m}\mu}$; (----) = radioactivity (cpm/0.2 ml fraction).

Results

Purification of Virus

The elution profile obtained during the chromatographic step of a purification of ^3H -Mengo virus by the procedure outlined in this chapter is shown in Figure 3.1. Evidence that this procedure yields virus from which at least 99.95% of contaminating cellular macromolecules is removed has been presented by Scraba et al. (1969).

Homogeneity of Viral RNA

The homogeneity of each preparation of RNA isolated from either highly purified ^3H -uridine labelled or unlabelled virions was assessed by sucrose gradient sedimentation analysis. Linear density gradients (4.5 ml) of 5-20% sucrose in PBS (containing 0.01 M EDTA) were prepared using a two-chamber mixing device (Buchler Instruments, Fort Lee, N.J.). The sample was layered on top of the gradient, which was then centrifuged (SW 39 rotor) for 3 hr at 35,000 rpm in a Spinco Model-L preparative ultracentrifuge. Fractions (0.2 ml) were collected dropwise from the bottom of the gradient, and diluted 1:10 with deionized water, for measurements of either $\text{O.D.}_{260\text{m}\mu}$ (unlabelled RNA) or radioactivity (^3H -uridine labelled RNA). Figure 3.2 illustrates the results of one such analysis of ^3H -Mengo RNA. The viral RNA, which is known from independent studies to have an intrinsic sedimentation

Table 3.1
Interaction of ³H-Mengo RNA and L Cells

Incubation medium ^a	Incubation time (min at 37°)	RNA taken up by 5x10 ⁶ cells ^b (% of added label)	Infectious centers produced per 5x10 ⁶ cells ^c
0.14 M PBS	5	0.28	900
	10	0.37	1,100
	15	0.45	700
0.14 M PBS containing 100 µg DEAE-D/ml	1	6.9	292,000
	3	6.7	363,000
	6	7.4	279,000
0.6 M sucrose/PBS	3	0.07	7,500
	6	0.09	12,500
	10	0.09	6,800
0.6 M sucrose/PBS containing 10% DMSO	3	0.07	17,500
	6	0.06	41,800
	10	0.06	23,900

^a The incubation media used in these experiments contained 1-2 µg ³H-Mengo RNA/ml and 1-2 x 10⁵ cpm/ml.

^b Values shown are the means of data from 6 separate experiments.

^c Illustrative data taken from one experiment in which uptake of ³H-RNA and infectious center formation were measured simultaneously.

coefficient of 35S (Scraba et al., 1967) sediments in the expected position with respect to the L cell 28S and 18S ribosomal RNA's, which were added to the sucrose gradient as an optical density marker.

Uptake of ^3H -RNA by L Cells from Various Incubation Media

The uptake of homogeneous, radiolabelled viral RNA by L cells during incubation in PBS, 0.6 M sucrose/PBS, 0.6 M sucrose/PBS-10% DMSO, and PBS containing 100 μg DEAE-D/ml was measured. The incubation times used for each medium were chosen to span the time which had been established as being optimal for the formation of infectious centers in that particular medium (see Figure 1.5). Table 3.1 summarizes the results of these experiments, the values shown being the means of values obtained from six separate experiments. The amount of RNA taken up by 5×10^6 cells is expressed as a percentage of the total RNA present per ml of incubation mixture. It should be emphasized that the amount of RNA "taken up" by cells is defined in these experiments as that RNA that remains bound to the cells after the latter are washed in growth medium, and that the use of this expression should not be interpreted as implying that all of this RNA actually penetrates the cell membrane.

Several points, illustrated by the data given in Table 3.1, are worth noting.

- (a) Although very few infectious centers are formed

in cell-Mengo RNA mixtures incubated in PBS, the amount of viral RNA taken up by cells from this medium is substantially greater than the amount taken up by cells incubated in the sucrose medium.

(b) The addition of 10% DMSO to the sucrose medium increases significantly the formation of infectious centers, (by a factor of approximately 4 in the experiment from which the data shown in Table 3.1 were taken) but does not increase the amount of viral RNA that becomes firmly cell-associated. If anything, it appears to decrease the uptake slightly.

(c) The presence of DEAE-D in the medium in which cells and RNA are incubated brings about a marked increase in the amount of viral RNA that becomes cell-associated. However, a comparison of infectious center formation and RNA uptake in PBS and PBS containing 100 μ g DEAE-D/ml makes it clear that the dramatic stimulation (approximately 400-fold) of infectious center formation produced by DEAE-D is not explicable on the basis of increased uptake alone, since RNA uptake is increased by a factor of only 20 in the presence of the polycation.

Uptake of 3 H-RNA by L, HeLa, and Human Diploid Cells

Although DMSO was found not to increase the uptake of viral RNA by L cells, the possibility remained that the much greater stimulation in infectious center production given by DMSO in the diploid cell-Mengo RNA system could be due, at least in part, to an increase in RNA uptake by

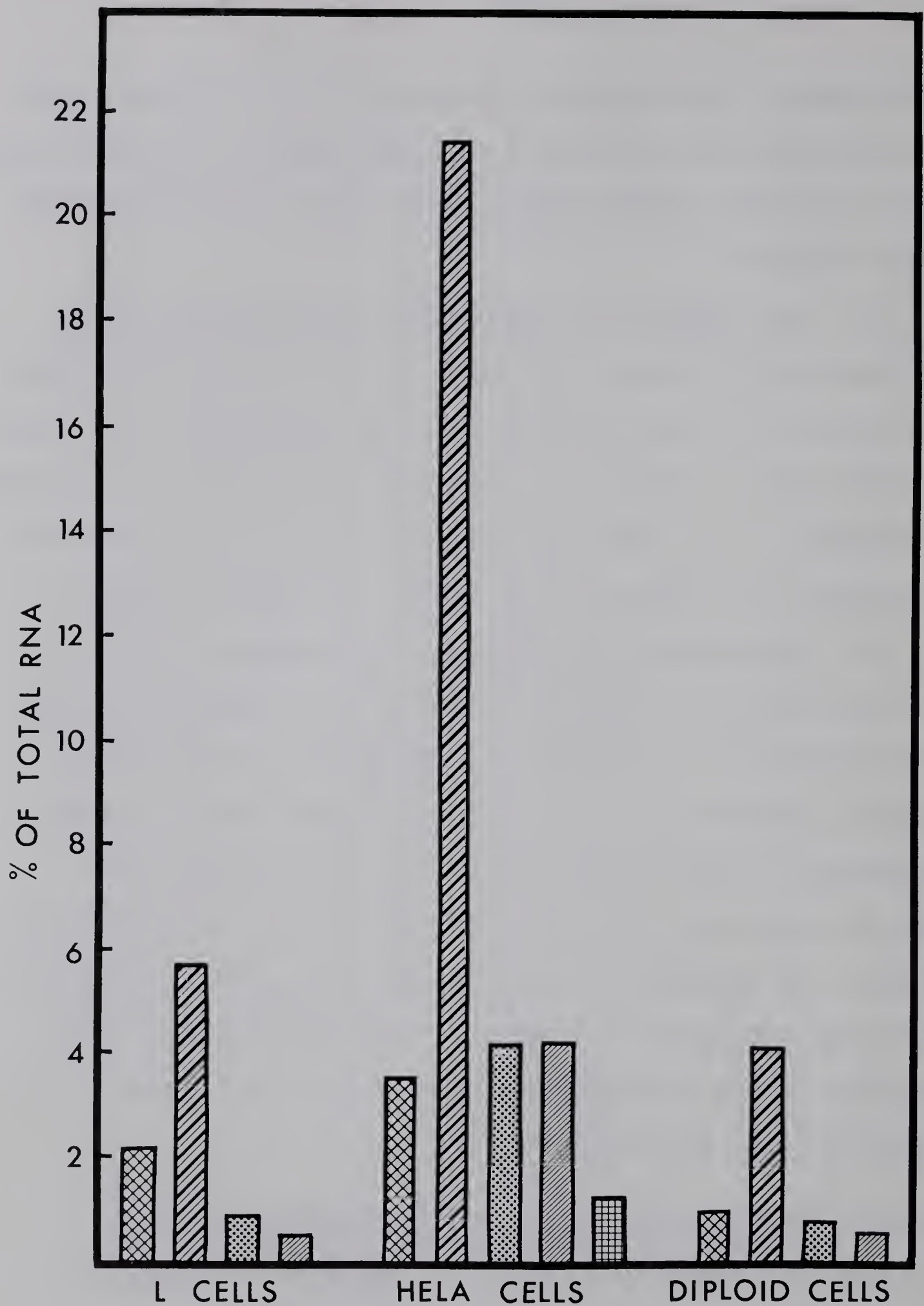


Figure 3.3. Uptake of ^3H -Mengo RNA by L, HeLa, and human diploid cells from various media (see Figure 2.15, facing page 66, for details regarding the media used).

these cells in the presence of this additive. A comparative uptake study, designed primarily to provide information regarding the uptake of ^3H -RNA by diploid cells from media containing DMSO, but, at the same time, to determine whether or not the relationship between uptake of RNA from, and infectious center formation in various incubation media was the same for L, HeLa, and diploid cells, was carried out. The uptake of ^3H -Mengo RNA by cells of the three types was measured simultaneously, using the incubation media that had been established as being optimal for infectious center formation with each cell type (i.e., the same media that were used in the comparative study of infectious center formation, the results of which are shown in Figure 2.15). The same RNA preparation was used in all mixtures, and the experimental procedure followed was the same as that described for the measurement of RNA uptake by L cells. The results of three separate experiments are illustrated in Figure 3.3. No significant difference between the amounts of radioactivity that became cell-associated during 5 and 10 minute periods of incubation in any medium was observed.

With each cell type, the greatest uptake of RNA was found to take place in the PBS-DEAE-D medium, but the uptake from this medium was not proportional to the number of infectious centers formed in L, HeLa, and diploid cells. The amount of RNA which became associated with diploid

cells in the sucrose/PBS-DMSO medium was no greater than the amount taken up by L cells from the same medium, although infectious center formation in this medium is much more efficient in diploid than in L cells. The uptake of ^3H -RNA from hypertonic salt solution by HeLa cells was lower than that taken up by the same cells from PBS; a phenomenon which was reported earlier by Borriss and Koch (1964). In all incubation media, the amount of ^3H -Mengo RNA taken up by HeLa cells was greater than the amount taken up by either L or diploid cells from the corresponding media, although, except in the case of PBS containing DEAE-D, the number of infectious centers formed was not. The results illustrated in Figure 3.3 support the conclusion derived from the corresponding studies with L cells alone, - namely that there is no constant correlation between the amount of RNA that becomes firmly cell-associated and the number of infectious centers formed. This lack of correlation is most strikingly exemplified by the behavior of the human diploid cells. With these cells, the greatest uptake of RNA was seen in PBS-DEAE-D, whereas the largest number of infectious centers was formed in the sucrose/PBS-DMSO medium.

Effect of Ribonuclease on L Cell-Associated ^3H -Viral RNA

Uptake experiments such as those that provided the data summarized in Table 3.1 and Figure 3.3, do not discriminate between ^3H -RNA that actually penetrates the cells and that which is adsorbed to the cell surfaces.

Table 3.2

Effect of RNase on Cell-Associated ^3H -Mengo RNA

Incubation medium ^a	cpm/ 10^7 cells				
	0 hrs		1 hr		3 hrs
		After RNase		After RNase	After RNase
<u>Exp. #1</u>					
PBS	670	640			
PBS-DEAE-D	35,210	4,550			
0.6 M sucrose/PBS	320	320			
0.6 M sucrose/PBS-DMSO	340	400			
<u>Exp. #2</u>					
PBS	740	620	600	540	
PBS-DEAE-D	40,120	4,340	13,560	4,980	
0.6 M sucrose/PBS	440	380	320	280	
0.6 M sucrose/PBS-DMSO	420	400	320	300	
<u>Exp. #3</u>					
PBS-DEAE-D	53,400	5,540	10,800	5,040	9,220 5,560
<u>Exp. #4</u>					
PBS	705	630	630	540	600 560
PBS-DEAE-D	43,690	5,620	10,060	5,250	10,080 5,560
0.6 M sucrose/PBS	380	350	320	280	320 300
0.6 M sucrose/PBS-DMSO	380	400	320	300	330 340

^a Cells were incubated, at a concentration of 10^7 cells/ml, in the indicated media. In all cases the media contained 660,000 cpm/ml (as ^3H -Mengo RNA). Incubation times were 10 min (PBS), 3 min (PBS-DEAE-D) and 6 min (0.6 M sucrose/PBS and 0.6 M sucrose/PBS-DMSO).

In an attempt to gain information regarding the localization of the cell-associated ^3H -RNA, a study was carried out to determine the proportion of that RNA that is resistant to degradation by pancreatic ribonuclease (RNase). The experimental design was as follows.

Aliquots of L cells were incubated with ^3H -Mengo RNA as in the uptake experiments, after which the suspensions were diluted 10-fold with growth medium, the cells sedimented by centrifugation and washed two times in 5 ml volumes of growth medium. One sample was then resuspended in 5 ml PBS, and the other in 5 ml PBS containing 5 μg RNase/milliliter. Both were incubated for 10 min at 37° , after which the suspensions were diluted to 10 ml with growth medium, the cells sedimented, washed in 5 ml of growth medium, and lysed by sonic vibration in 1 ml volumes of distilled water. At the same time, other duplicate cell samples, after being incubated with ^3H -Mengo RNA and washed in growth medium, were resuspended in growth medium and incubated at 37° for periods of one or three hours before being treated with RNase as outlined above. The results of these experiments are summarized in Table 3.2.

Virtually none of the RNA that became cell-associated during incubation in PBS or in either of the sucrose media was found to be removed from the cells by treatment with RNase. This was so both with cells treated with enzyme immediately after exposure to the ^3H -RNA and with cells in which treatment was delayed for one or three hours. In

the case of cells incubated in the PBS-DEAE-D medium, approximately 90 per cent of the label was shown to be removed when the cells were treated with RNase immediately after exposure to ^3H -RNA. When such cells were incubated in growth medium after exposure to ^3H -RNA, it was found that a large proportion (up to 75%) of the label eluted spontaneously from the cells, although the amount of "RNase-resistant" ^3H -RNA remained remarkably constant.

If one makes the assumption that the ^3H -RNA that is not removed from the cells by RNase treatment is located intracellularly, these data suggest that essentially all of the RNA that becomes cell-associated in the PBS or sucrose media actually penetrates the cell membrane. It seems clear that most of the RNA that becomes cell-associated in the presence of DEAE-D is merely adsorbed to the cell surface, from which it can be displaced readily by treatment with RNase, and from which it may elute spontaneously.

Physical Parameters of Unabsorbed Viral RNA

Attempts were made to learn something of the physical parameters of the viral RNA which remains extracellular after incubation with L cells in the four media used in the uptake studies. In these experiments, ^3H -Mengo RNA was incubated with cells, after which the cells were removed by centrifugation, and the sedimentation behavior of unabsorbed viral RNA in sucrose density gradients was examined. Incubation was for 10 min at 37° , at a cell

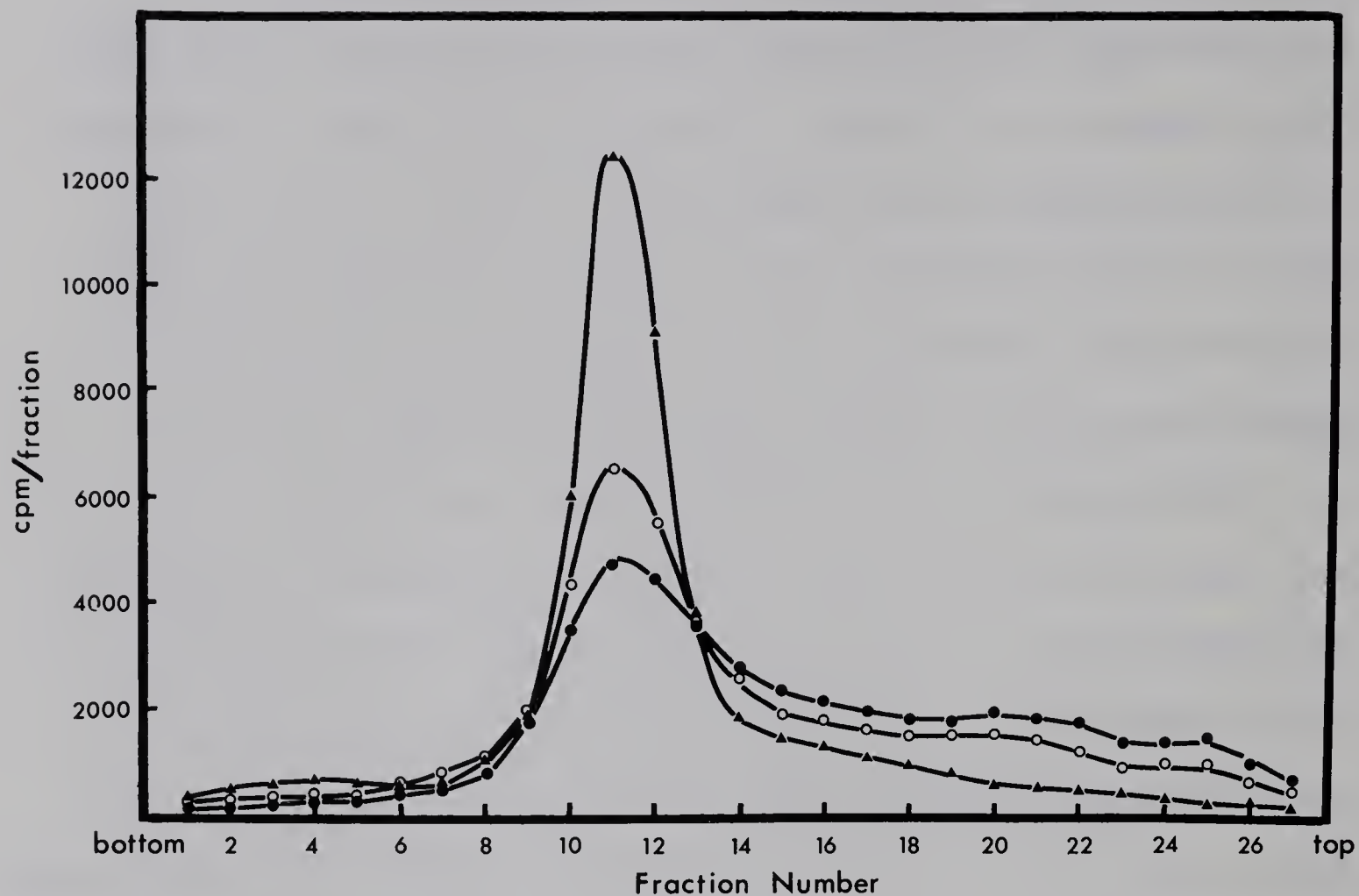


Figure 3.4. Sucrose density gradient (5-20%) sedimentation analysis of ^3H -Mengo RNA.

- ▲ = control (incubated in absence of cells)
- = incubated with L cells in 0.6 M sucrose/PBS
- = incubated with L cells in 0.6 M sucrose/PBS-10% DMSO

Centrifugation was for 2.5 hr at 35,000 rpm in a Spinco SW39 swinging bucket rotor.

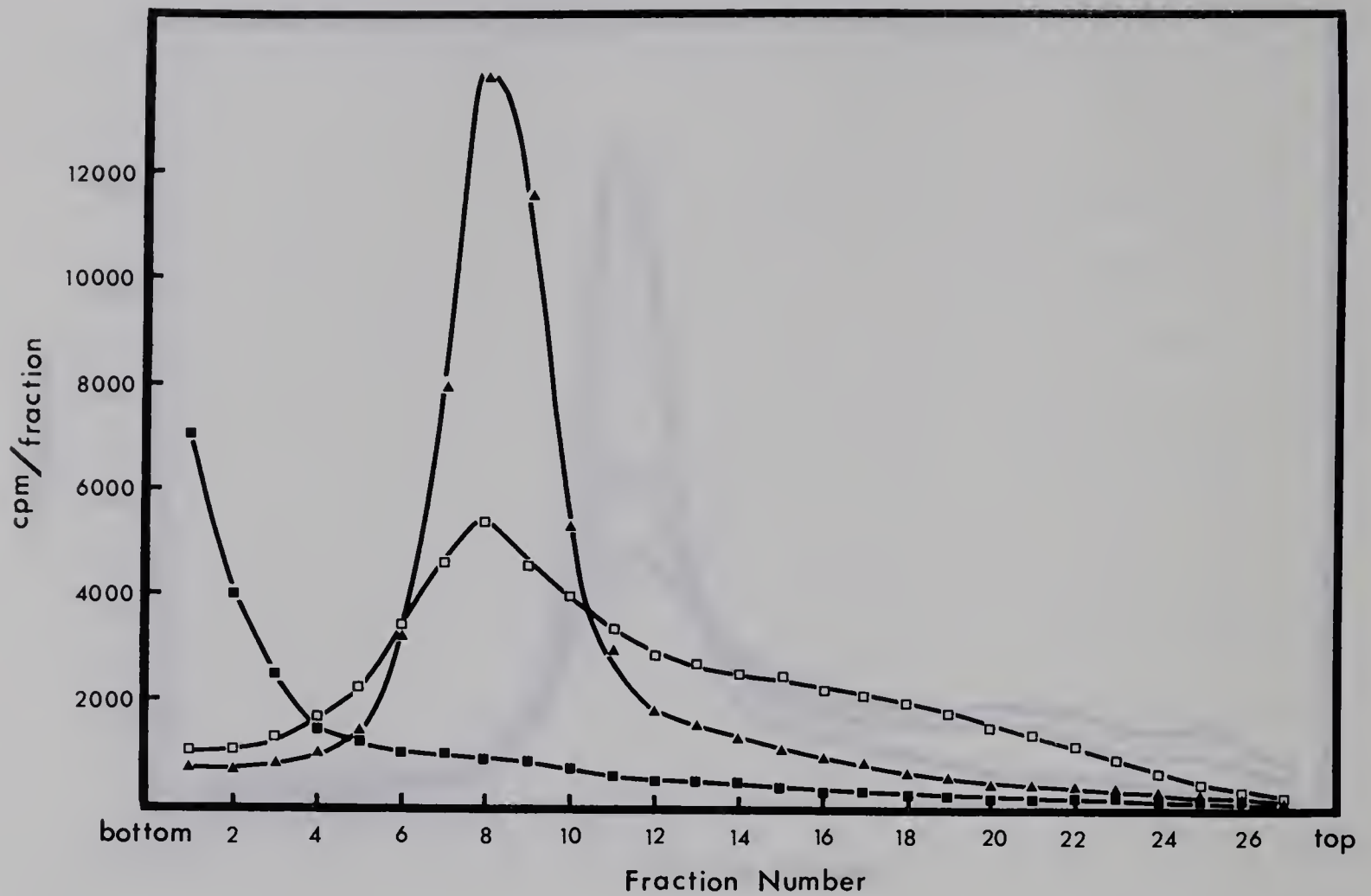


Figure 3.5. Sucrose density gradient (5-20%) sedimentation analysis of ^3H -Mengo RNA.

- ▲ = control (incubated in the absence of cells)
- = incubated with L cells in PBS
- = incubated with L cells in PBS containing 100 μg DEAE-D/ml

Centrifugation was for 3 hr at 35,000 rpm in a Spinco SW39 swinging bucket rotor.

concentration of 5×10^6 /milliliter. The results of these studies are illustrated in Figures 3.4 and 3.5.

It is clear that, although some undegraded 35S material remained, there was fairly extensive degradation of the viral RNA in the PBS, 0.6 M sucrose/PBS and 0.6 M sucrose/PBS-10% DMSO media. The degradation was perhaps somewhat more extensive in PBS than in either of the sucrose media (although the difference was not marked), and appeared to be more extensive in the presence of DMSO than in its absence. In the case of the sample incubated in the medium containing DEAE-D, virtually no radioactivity was found in the gradient. The viral RNA, (undoubtedly complexed with DEAE-D as will be discussed further in a later section of this chapter) was sedimented to the bottom of the gradient.

A related study involved an examination of the rate of degradation of RNA (in this case a mixture of 18S and 28S ribosomal RNA's from L cells) by pancreatic ribonuclease in these same four media. It was found that the rate of degradation of RNA to acid-soluble fragments was most rapid in 0.6 M sucrose/PBS-10% DMSO. This result was not entirely unexpected, since DMSO has been shown to increase the activity of certain enzymes, among which is pancreatic deoxyribonuclease (Monder, 1967). The rates of hydrolysis in the other three media were found to be essentially identical. There was no indication that DEAE-D, at a concentration of 100 μ g/ml, protected the RNA from degradation

by the nuclease, - again a not unexpected finding in view of the reports by Pagano et al. (1967) and Maes et al. (1967) that RNA is only partially protected against ribonuclease degradation by much higher concentrations of DEAE-D than those used in this study.

Physical Parameters of Viral RNA Taken Up by Cells

Repeated attempts to isolate undegraded, 35S viral RNA from L cells which had been incubated with ³H-Mengo RNA in each of the four media (PBS, PBS containing 100 µg DEAE-D/ml, 0.6 M sucrose/PBS, and 0.6 M sucrose/PBS-10% DMSO) were unsuccessful. The procedure used was as follows.

Washed L cells were incubated at 37° with ³H-labelled Mengo RNA in each of the media used in this study for an appropriate length of time (5, 10, and 15 min in PBS-DEAE-D, sucrose or sucrose-DMSO, and PBS respectively), after which the mixtures were diluted with growth medium, and the cells were recovered by centrifugation. The cells were then washed once with growth medium, resuspended in growth medium and divided into 3 aliquots each containing 5×10^7 cells. One aliquot was centrifuged, the cells washed once in PBS containing 0.05% BPA and the RNA was extracted therefrom immediately. The other two were incubated at 37° - one for 30 min and the other for 2 hr - before being processed in the same way. For isolation of the RNA, the washed cells were suspended in 4.5 ml of buffer, pH 7.4, consisting of 0.01 M Tris, 0.1 M NaCl, and 0.001 M EDTA (TNE),

and the suspension was added to a tube containing 0.5 ml of a 10% SDS solution, 0.05 ml of a 2% Macaloid suspension, and 5 ml TNE-saturated phenol. The contents of the tube were mixed vigorously (Lab-line super mixer) at room temperature for 10 min, chilled in ice, and centrifuged at 2000 g for 10 min at 4⁰. The aqueous layer was carefully removed, and extracted one more time with buffer-saturated phenol. The RNA was precipitated from solution by the addition of 2 volumes of ethanol and 0.1 volume of a 20% solution of potassium acetate (to ensure complete precipitation, the mixture was allowed to stand overnight at -20⁰). The samples of RNA, collected by centrifugation, were washed once in 75% ethanol, and dissolved in small volumes (0.3-0.5 ml) of TNE. Aliquots (0.2 ml) of the solutions were layered on 5-20% sucrose (in TNE) gradients, which were then centrifuged for 3 hr at 35,000 rpm in the SW39 rotor of a Spinco Model L ultracentrifuge. The distribution of ultraviolet (254 m μ) absorbing material in the gradients was measured using an ISCO Model D density gradient fractionator and Model UA-2 ultraviolet analyzer (Instrumentation Specialties Company, Inc., Lincoln, Nebraska). Fractions of 5 drops each (approximately 0.17 ml) were collected directly on filter paper discs. The latter were then treated with TCA as described earlier, and the amount of radioactivity on each was measured in a Beckman liquid scintillation spectrometer.

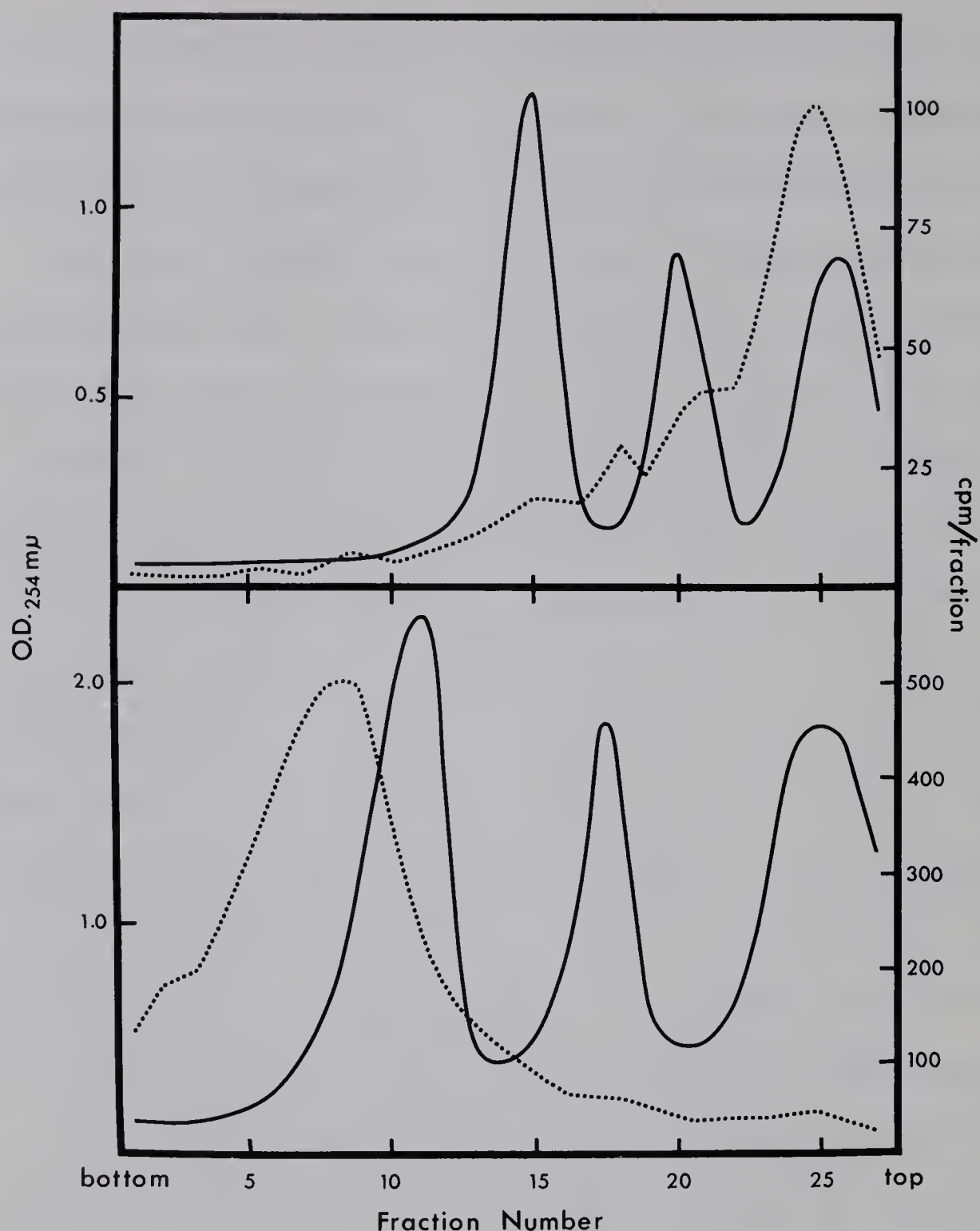


Figure 3.6. Sucrose density gradient (5-20%) sedimentation analysis of the total RNA isolated from L cells infected with ^3H -Mengo RNA or ^3H -Mengo virus.

Upper panel: RNA isolated from 5×10^7 L cells 2 hr after they were incubated with ^3H -RNA in 0.6 M sucrose/PBS. Centrifugation was for 3 hr at 35,000 rpm.

Lower panel: RNA isolated from 5×10^7 L cells 2 hr after they were incubated with ^3H -virus. Centrifugation was for 3.5 hr at 35,000 rpm.

(—) O.D. (254 mμ) ; (----) radioactivity

A large number of experiments of this type were carried out. Invariably, most of the radiolabel was found at the top of the gradient, with the remainder of the counts distributed throughout the rest of the gradient. In no case was a peak of radioactivity found in the position at which a molecule of sedimentation velocity 35S would band. This result was obtained regardless of when the RNA was isolated (either immediately after incubation with ^3H -Mengo RNA or after an additional period of incubation of 30 min or 2 hr), and regardless of the medium in which cells and ^3H -Mengo RNA were incubated. The total amount of radioactivity recovered, did, of course, differ from one incubation medium to the other (PBS-DEAE-D > PBS > sucrose), but the profiles (plots of cpm vs fraction number) did not differ qualitatively one from the other. Illustrative data are shown in the upper panel of Figure 3.6. The RNA sample, in this particular case, was isolated from L cells two hours after they had been incubated with ^3H -Mengo RNA in 0.6 M sucrose/PBS. The optical density profile is, of course, given by the cellular RNA's present.

It was considered possible that these results did not reflect accurately the events that were occurring in the infected cells, and that the viral RNA was degraded during the extraction procedure. In an effort to resolve this question, two different experiments were carried out. First, a small volume of a solution of ^3H -Mengo RNA was added to a cell suspension, and the mixture was immediately

added to an equal volume of buffer-saturated phenol. The total RNA was extracted, precipitated, and analyzed by sucrose density gradient centrifugation by the procedures described above. The radioactivity profile obtained from the sucrose gradient analysis of the isolated RNA showed that while some of the label sedimented in the upper half of the gradient, most of the ^3H -RNA was found in the position where intact, 35S RNA would be expected. In a more extensive study, cells were incubated (at a multiplicity of infection of 10) with highly purified ^3H -uridine-labelled Mengo virus, washed to remove unattached virus, suspended in spinner medium and allowed to incubate at 37° for periods of from 0 to 4 hours. At intervals, RNA was extracted from aliquots of 5×10^7 cells, precipitated, and analyzed by sucrose density gradient sedimentation using procedures identical to those used with cells infected with naked ^3H -Mengo RNA. The radioactivity profiles obtained with RNA samples isolated at 0, 1, 2, 3 and 4 hours post-infection, were found to be qualitatively very similar, - as were profiles obtained with RNA samples isolated at various times after infection with ^3H -Mengo RNA. Illustrative data, presented in the lower panel of Figure 3.6, show clearly that most of the ^3H -RNA recovered from ^3H -virus infected cells sedimented to the expected position for intact 35S viral RNA. These results make it very clear that it is possible, using the procedure

described here, to isolate intact, viral RNA (i.e., the infecting genomes) from cells infected with intact virus particles, and that the degradation of viral RNA that occurs during isolation is minimal. The repeated failure to do so in the case of cells infected with viral RNA suggests that most of the RNA taken up by cells in the latter system(s) is rapidly and extensively degraded.

From a qualitative point of view, the length of time that cells infected with either ^3H -Mengo RNA or ^3H -Mengo virus were incubated at 37° before isolation of the RNA had no effect on the data obtained. However, in both cases, the total number of counts recovered decreased progressively as the incubation time was increased. In the case of virus-infected cells, and also those infected with RNA in the presence of DEAE-D, a reasonable explanation of this phenomenon could be that a proportion of the virus particles or RNA-DEAE-D complexes that became attached to the cells failed to penetrate the cell membrane and eluted therefrom during the incubation period. An alternate explanation might be that, in both cases, the viral RNA entered into some cellular structure from which it was not released by the isolation procedure used here. The latter does not seem a particularly likely possibility, since phenol extraction at elevated temperatures (60°) did not increase the amount of ^3H -RNA recovered from virus-infected cells.

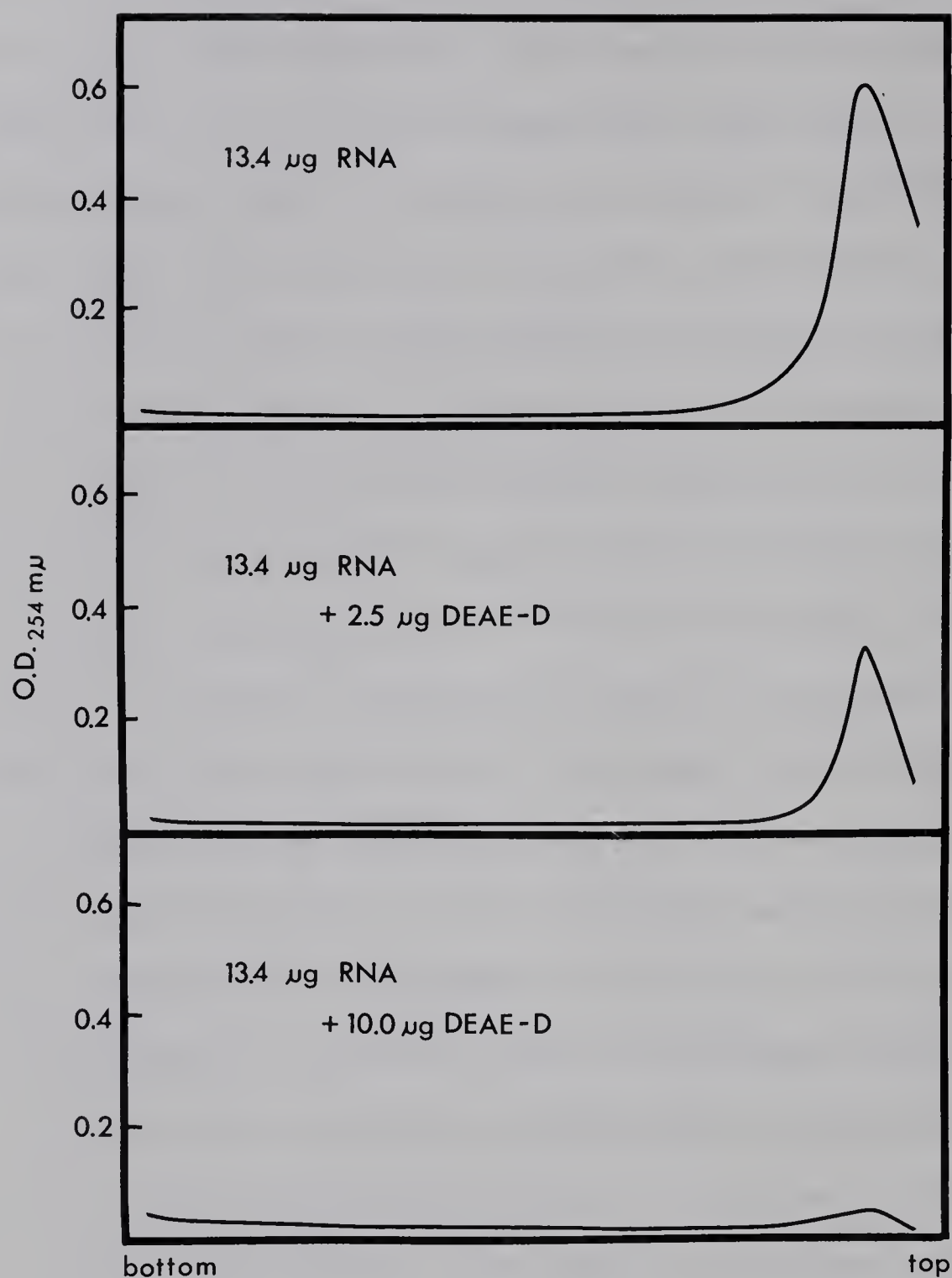


Figure 3.7. Sucrose density gradient (15-40%) sedimentation analysis of RNA-DEAE-D mixtures. Solutions containing the amounts of RNA and DEAE-D indicated in the figure were layered on each of three gradients which were centrifuged for 1 hr at 20,000 rpm in a Spinco SW39 swinging bucket rotor. The O.D. profiles were determined using an ISCO Model D density gradient fractionator and Model UA-2 ultra-violet analyzer.

Association between DEAE-D and RNA Molecules

The observation, illustrated in Figure 3.5, that the ³H-Mengo RNA not absorbed by cells in incubation mixtures containing DEAE-D sedimented to the bottom of a sucrose gradient, suggested that the unabsorbed RNA might be part of a larger structure, probably a complex of RNA and DEAE-D molecules. In an attempt to determine whether a complex of uniform composition was formed, and if so, to determine the size of the complex, mixtures of RNA and DEAE-D were centrifuged in sucrose density gradients under a variety of conditions. Under no condition was the complex found to form a band in the gradient. Even centrifugation at 10,000 rpm for 30 min in a 15-40% sucrose gradient was sufficient to sediment all of the RNA to the bottom of the tube. These results suggest strongly that very large complexes must be formed:- a complex consisting of one molecule of each of the two polymers, for example, should be readily banded in a sucrose density gradient. The results of another study, illustrated in Figure 3.7, provided evidence that such complexes consist of a number of molecules of each of RNA and DEAE-D, and that the ratio of RNA to DEAE-D therein may not be constant. From this figure, it may be seen that when a mixture contained approximately equal numbers of RNA and DEAE-D molecules, all of the RNA became associated with DEAE-D to form larger, rapidly sedimenting structures (bottom panel). However, as shown in the center panel of the figure, when

RNA and DEAE-D were present in a ratio of 4:1, approximately half of the RNA remained unassociated with DEAE-D although those RNA molecules that did complex with the polycation were still sedimented to the bottom of the tube. These observations suggest that in both cases, the complexes formed consisted of multiple strands of the two polymers, but that the ratios of RNA:DEAE-D in the complexes were of the order of 1:1 and 2:1 respectively. The results of these studies, in which a much higher RNA:DEAE-D ratio was used than was employed in either the infectivity studies (usually less than 1:100) or the uptake studies (at most 1:10), suggest that the concentration of DEAE-D (relative to that of RNA) necessary for optimal infectious center production is much higher than that necessary for complexing all of the infectious viral RNA.

Interaction between DEAE-D, RNA, and Cells

Evidence suggesting that DEAE-D can bind to cells in the absence of RNA has been presented by other investigators. Bachrach (1966) reported that pre-washing calf kidney cell monolayers with a solution containing 1000 μ g DEAE-D/ml before adding the viral RNA thereto enhanced the sensitivity of the assay of FMD RNA to about the same extent as was observed when DEAE-D (at the same concentration) was present in the RNA solution added to the monolayers. Pagano and Vaheri (1965) incubated monolayers of MK cells with a solution containing 1000 μ g DEAE-D/ml, and then washed them

three times before inoculating them with polio RNA. They found that this procedure stimulated the infectivity of the polio RNA to some extent, but that the effect was much less than that produced by the simultaneous addition of DEAE-D and RNA to the cell monolayers. In the present study, DEAE-D (at a final concentration of 100 or 500 $\mu\text{g}/\text{ml}$) was added to L cells in suspension. The cells were removed by centrifugation, washed once, and were then incubated with Mengo RNA in PBS in the absence of DEAE-D. Very little, if any, increase in infectious center formation compared with that obtained in control, untreated cells was found. The differences among the results obtained from these three investigations may simply reflect differences in the amount of DEAE-D that remained in association with the assay cells in the three cases, assuming that the association is reversible, and that DEAE-D may be washed more efficiently from suspended cells than from cells in monolayers. Evidence which shows that DEAE-D does bind to suspended cells was obtained by incubating cells in a solution of PBS containing 100 μg DEAE-D/ ml , removing the cells by centrifugation, and using the supernatant solution as the incubation medium for the assay of RNA with fresh cells. Only one-fifth as many infectious centers were formed in this medium as were produced in the same number of cells incubated with the same concentration of the same RNA in PBS containing 100 μg

DEAE-D/ml, which suggests that much of the DEAE-D had been removed from the medium that had been preincubated with cells.

The effect on infectious center formation of varying the sequence in which RNA, DEAE-D, and L cells were added to the incubation mixture was investigated. The results showed clearly that the infectious process is most efficient when the RNA-DEAE-D complex is formed as the first step. Substantially fewer infectious centers were formed when either DEAE-D or RNA was added to suspended cells which had already been incubated for 5 min with RNA or DEAE-D respectively, than were produced when RNA and DEAE-D were mixed 5 min prior to their addition to the cells. These data were interpreted as evidence that the formation of the RNA-DEAE-D complex was much less efficient if cells were present to compete with the RNA for binding to DEAE-D.

Factors which Influence the Efficiency of the RNA Assay

A study in which both uptake of ^3H -Mengo RNA by, and infectious center formation in successive L cell samples incubated in the same RNA solution was carried out. Three incubation media - PBS containing 100 μg DEAE-D/ml, 0.6 M sucrose/PBS, and 0.6 M sucrose/PBS-10% DMSO, were examined, and the experimental procedure followed was essentially identical to that described in Chapter 1. Each cell sample, after incubation in the ^3H -RNA solution, was resuspended in growth medium. An aliquot was removed and titrated for

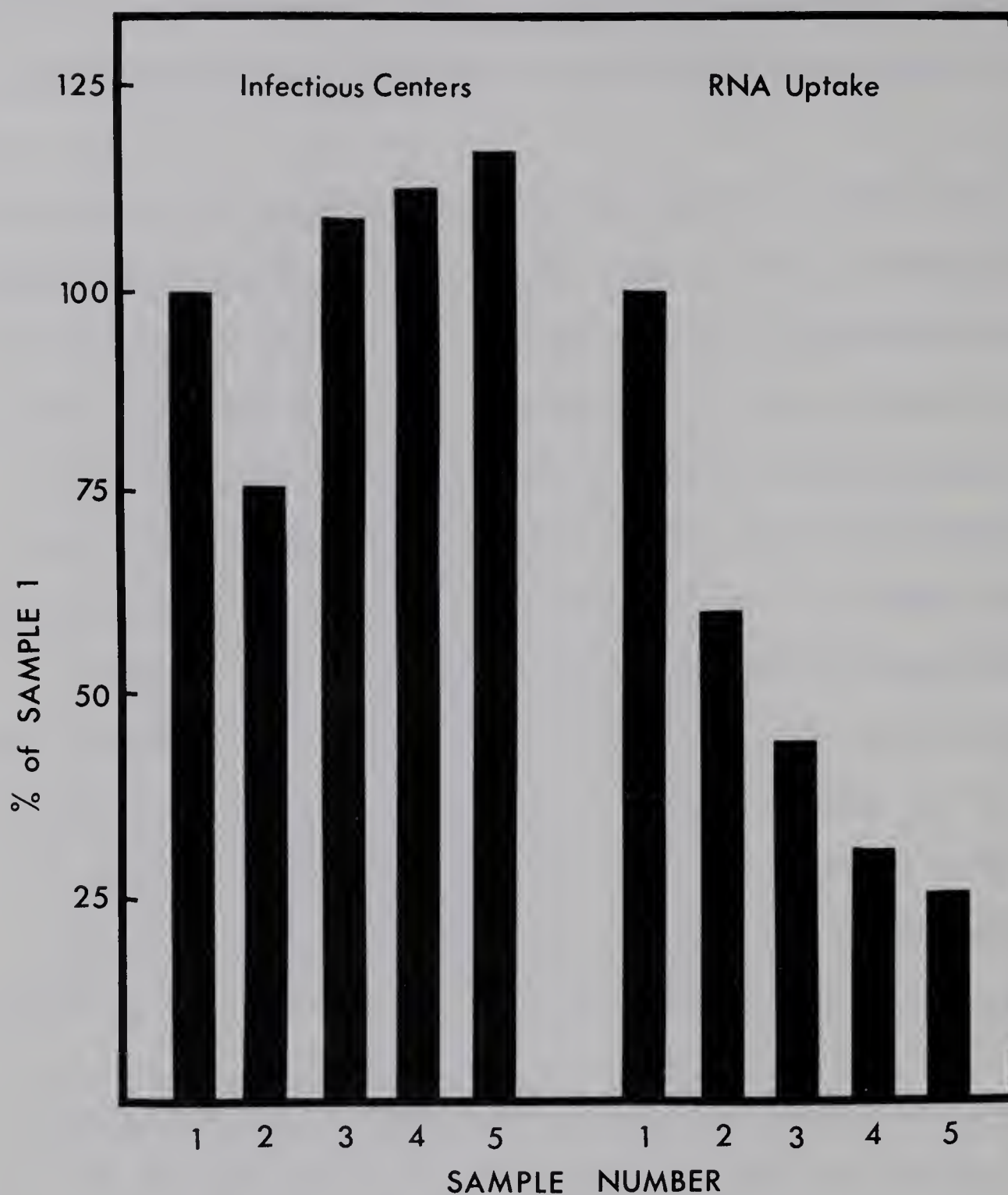


Figure 3.8. Infectious center formation and ^3H -RNA uptake in successive cell samples incubated at 37° for 3 min in the same RNA-PBS-DEAE-D solution.

infectious centers in the usual manner, and the remaining cells in each sample were washed, disrupted by sonic vibration, and the radioactivity present in each sonicate was measured. All incubation media contained approximately $20 \mu\text{g } ^3\text{H-Mengo RNA/ml}$, a concentration that gave a level of radioactivity of about 3×10^5 cpm per milliliter. In each of 0.6 M sucrose/PBS and 0.6 M sucrose/PBS-10% DMSO, the number of infectious centers produced in, and the amount of $^3\text{H-RNA}$ taken up by each of five successive cell samples were found to be essentially the same. However, although the number of infectious centers formed in each of five successive cell samples incubated in the RNA-PBS-DEAE-D solution (an additional $500 \mu\text{g}$ of DEAE-D was added to each of samples 2-5, see Figure 1.11) was the same, the amount of radiolabel taken up decreased with each succeeding cell sample (the amount taken up by sample 5 was only about 25% of that taken up by sample 1). These rather surprising results, illustrated in Figure 3.8, suggest that more RNA is taken up by cells under the conditions used here than is required for maximum infectious center production in a given number of cells.

Subsequently, the relationship between the concentration of RNA and the number of infectious centers formed in a fixed number of cells incubated in PBS containing $100 \mu\text{g DEAE-D/ml}$ was determined. The number of infectious centers produced was found to increase linearly with increasing RNA

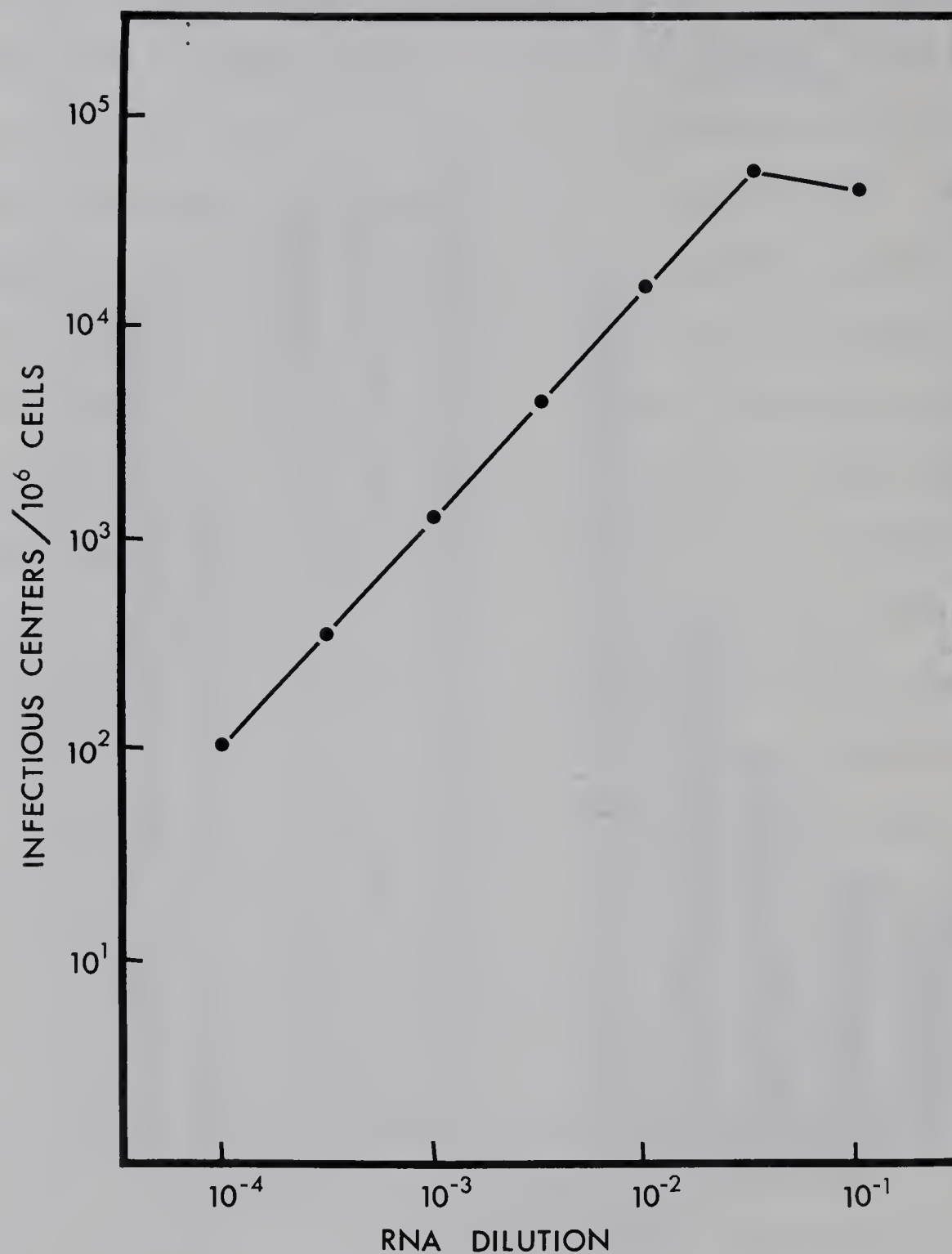


Figure 3.9. Relationship between infectious center formation and concentration of RNA. Incubations were carried out at 37° in PBS containing 100 μ g DEAE-D/ml and at a cell concentration of 2.5×10^6 /milliliter. The undiluted RNA solution contained 21.6 μ g/milliliter.

concentration up to a concentration of 1 $\mu\text{g}/\text{milliliter}$. At concentrations of RNA above 1 $\mu\text{g}/\text{ml}$, no further increase in the number of infectious centers formed/ 10^6 cells was obtained, although even at these RNA concentrations, less than 10% of the cell population produced virus plaques (i.e., registered as infectious centers) on cell monolayers. These results are illustrated in Figure 3.9. The experiment was repeated using three different concentrations of DEAE-D (50, 100 and 500 $\mu\text{g}/\text{ml}$), and the same result was obtained at all three polycation concentrations.

These results would seem to provide an explanation for the phenomenon illustrated in Figure 3.8, since in those experiments, the RNA concentration in the incubation media remained well above 1 $\mu\text{g}/\text{ml}$, even after incubation with five successive cell samples. It is likely, therefore, that the maximum number of infectious centers were produced in each cell sample.

Another interesting study was carried out, the results of which support the premise that the amount of RNA taken up from PBS-DEAE-D solutions is only one of the factors which determine the efficiency of infectious center formation. Pagano et al. (1967) speculated that at low concentrations of the polycation, RNA and DEAE-D may form complexes that can bind to cells, but that higher DEAE-D concentrations may be necessary to damage the cell membranes sufficiently to stimulate uptake of the complexes. In order

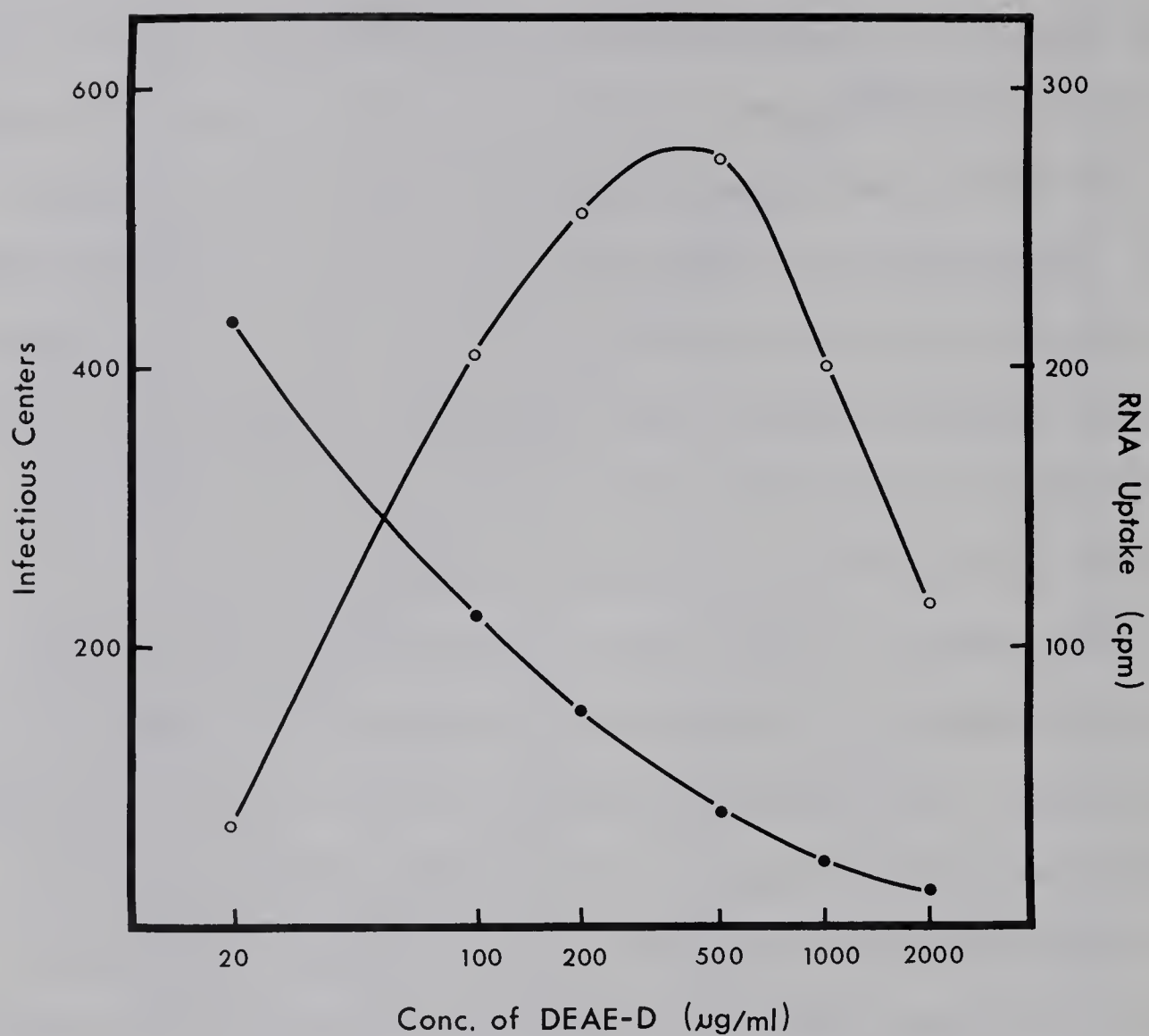


Figure 3.10. The effect of DEAE-D concentration on the number of infectious centers formed in, and the amount of RNA taken up by 10^6 L cells. Each incubation medium contained ^3H -RNA at a final concentration of $0.5 \mu\text{g/ml}$ and 10^5 cpm/milliliter. (o) infectious centers; (●) RNA uptake.

to test this proposition, experiments were carried out in which the uptake of ^3H -Mengo RNA, infectious center formation, and cell viabilities were measured simultaneously in L cells incubated with aliquots of an RNA solution containing DEAE-D at concentrations ranging from 20-2000 $\mu\text{g}/\text{milliliter}$. The rather surprising results are summarized in Figure 3.10, from which it may be seen that while maximum uptake occurred at a DEAE-D concentration of 20 $\mu\text{g}/\text{ml}$, maximum infectious center formation was observed at a polycation concentration of 250-500 $\mu\text{g}/\text{milliliter}$. The percent of cells that remained viable (as determined by their ability to exclude erythrosin stain) was found to be relatively constant (80-90%) over the range of DEAE-D concentrations, 20-1000 $\mu\text{g}/\text{milliliter}$. A substantial decrease in viability (to 50-60%) was seen in those cells incubated in the solution containing 2000 μg DEAE-D/ milliliter . There was, however, no obvious correlation between the percent of cells that remained viable and either the amount of ^3H -RNA taken up or the number of infectious centers formed.

The Production of Virus in L Cells Infected with Mengo RNA

It was observed, throughout the studies described in Chapters 1 and 2, that the plaques produced by infectious centers formed in PBS-DEAE-D were smaller than those produced by infectious centers formed in sucrose/PBS-DMSO solutions. To determine whether these differences in

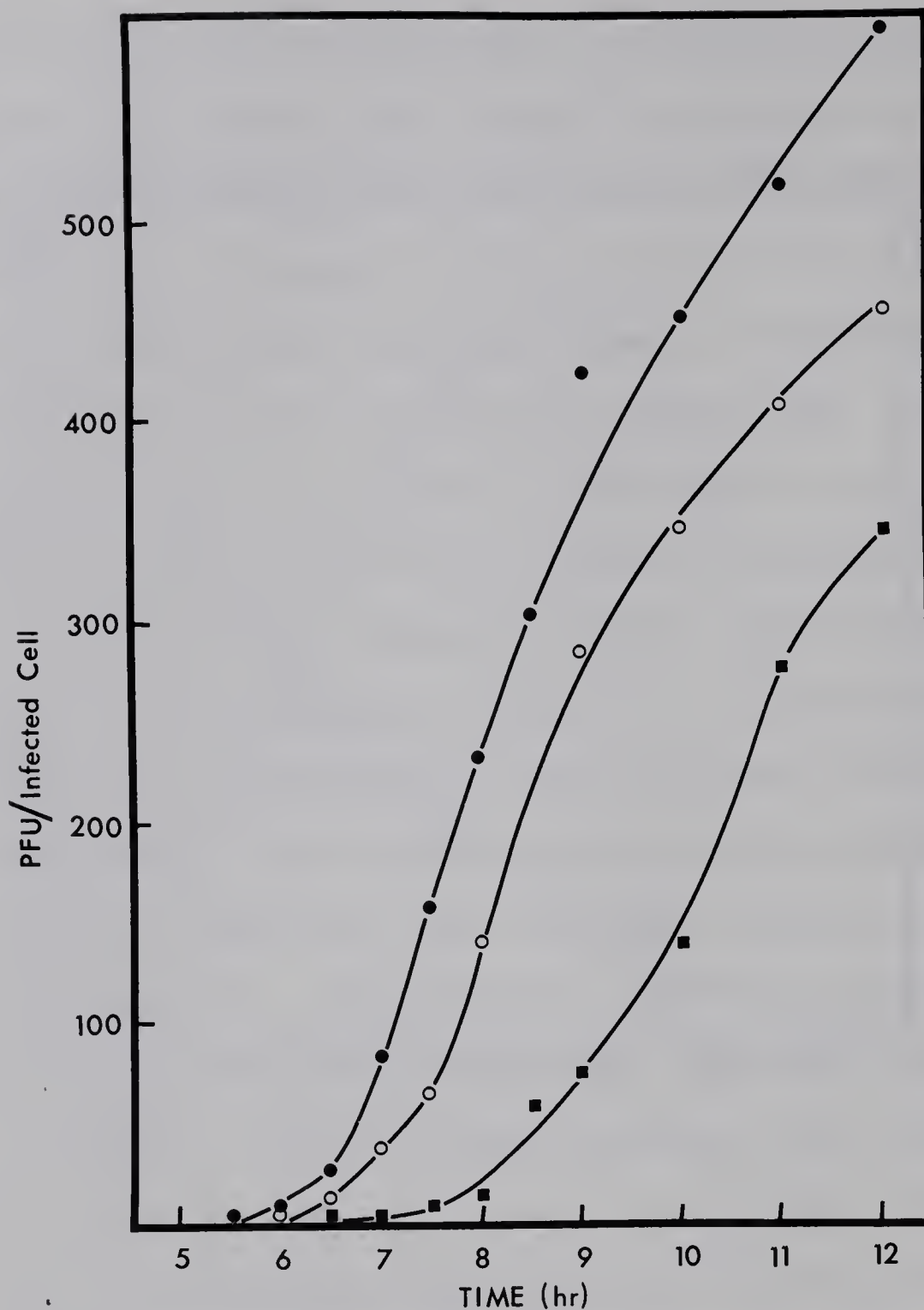


Figure 3.11. Single cycle growth curves obtained with L cells infected with Mengo RNA. Cells were infected in (●) 0.6 M sucrose/PBS-10% DMSO, (○) 0.6 M sucrose/PBS, and (■) PBS containing 100 µg DEAE-D/ml, then added to pre-warmed spinner medium, and maintained in suspension culture.

plaque size were the result of differences in either the number of infectious virus particles produced per infected cell, or in the length of the lag period before production of progeny virus began, single cycle growth studies were carried out as described below.

Washed L cells were suspended (at a concentration of 5×10^6 cells/ml) in 0.6 M sucrose/PBS, 0.6 M sucrose/PBS-10% DMSO, and PBS containing 100 μ g DEAE-D/ml, - each solution containing the same concentration of Mengo RNA. After incubation at 37° for 6 min (sucrose solutions) or 3 min (PBS-DEAE-D), an aliquot of each cell suspension was diluted 1:10 into growth medium and titrated for infectious centers in the usual way. The remainder of each cell sample was added immediately to a spinner flask containing pre-warmed (37°) medium (final cell concentration = 10^5 cells/ml), and incubation at 37° was continued. At intervals, a 1 ml aliquot was removed from each spinner flask and added to 4 ml cold virus diluent in 12 ml centrifuge tubes. The contents of each tube were frozen and thawed twice to disrupt the cells, the samples were centrifuged to remove cellular debris, and the supernatant solutions thus obtained were titrated for infectious virus. The results, illustrated in Figure 3.11, are expressed as plaque forming units per infected cell in order to give a more valid comparison of the time course of virus production in the three samples. It is clear that virus production begins somewhat earlier

in cells infected with RNA in 0.6 M sucrose/PBS-10% DMSO than in those infected in 0.6 M sucrose/PBS, and that virus production is delayed in cells infected in the presence of DEAE-D. If, as seems altogether likely from studies described earlier, viral RNA is taken up from this medium as a complex with DEAE-D, and if it were released from this complex slowly and asynchronously (and assuming that it could function as a messenger only after release from the complex), one would expect not only that the initiation of virus production would be delayed, but that the slope of the growth curve would not be as steep as it would be if all the RNA were able to exercise its biological functions immediately upon entering the cell. No direct evidence is available to support this theory, but at present it appears to be the most likely explanation for the observations shown in Figure 3.11. These data were obtained in a single experiment. A second experiment confirmed these results with respect to the times at which virus production begins in cells infected in the three media. In both experiments, burst sizes were calculated, and in both the burst size obtained with cells infected in 0.6 M sucrose/PBS-10% DMSO, was larger (by about 50%) than that obtained with cells infected in 0.6 M sucrose/PBS. In one experiment comparable burst sizes were obtained with cells infected in 0.6 M sucrose/PBS and PBS-DEAE-D, while in the other experiment, the burst size of cells infected in the latter medium was lower.

Discussion

Although the information obtained from the studies described in this chapter have not made it possible to define the precise mechanisms by which either sucrose solutions of elevated osmolarity or the additives, DMSO and DEAE-D, stimulate the formation of infectious centers in suspended cell-Mengo RNA mixtures, they have provided some insight into a number of aspects of the interaction between cultured cells and viral RNA.

Some years ago, it was shown that the measurable infectivity of viral RNA preparations could be increased by incubating the RNA and assay cells in media of elevated tonicity or osmolarity. Several possible explanations for this finding were advanced, including those that more RNA was taken up by cells from hypertonic than from isotonic medium, and that the activity of extracellular and/or cell membrane-associated ribonucleases were inhibited in solutions of elevated ionic strength. However, results obtained in the present study, in agreement with those reported by Borriss and Koch (1964), showed clearly that more RNA was taken up by cells from PBS than from solutions of elevated osmolarity. Also the viral RNA that did not become cell associated, appeared to be protected very little, if at all, in sucrose solutions against degradation by extracellular or membrane nucleases. In fact, degradation of the viral RNA by extracellular or membrane-associated

nucleases did not seem to be an important factor in limiting the number of infectious centers formed in either PBS or 0.6 M sucrose/PBS (with or without DMSO), since in all cases a significant amount of intact, infectious RNA remained in the medium after incubation with cells. In contrast to these observations, Bases and Huppert (1966) reported that, while about 2% of the labelled EMC RNA incubated with Krebs II cells in sucrose medium was adsorbed to the cells, the remainder was rapidly degraded. They were unable to detect any infectivity in the solution after removal of the cells, and the unadsorbed RNA was found to sediment in the upper region of a sucrose density gradient under conditions of centrifugation which would band intact viral RNA molecules near the bottom of the gradient. The differences between the findings of these investigators and those described in this thesis may be due to the fact that different cell types (possibly differing with respect to levels of cell-associated nucleases) were used in the separate studies. It may also be worth noting that a significant proportion of the input viral RNA preparation used by Bases and Huppert was rather extensively degraded.

Although in the L cell-Mengo RNA system the amount of degradation due to extracellular nucleases does not appear to be a major factor in determining the number of infectious centers formed in various incubation media, the extent to which the RNA that is taken up is degraded at the

intracellular level very likely is important. Colter and Ellem (1961b) suggested that sucrose solutions of elevated osmolarity may exert their stimulatory effect by dehydrating the cells, and that this might in turn, by creating a hypertonic intracellular environment, inhibit the activity of intracellular nucleases. Alternatively, the effect of sucrose might be related to the formation of large vacuoles at the periphery of the cells,- this being a common cellular response to a variety of adverse conditions. Points of rupture of the vacuolar membranes in cells exposed to sucrose have been described (Trump and Janigan, 1963), and this may provide a route of entry by which RNA molecules could escape digestion before reaching the cell cytoplasm. It has been suggested that the digestive (degradative) functions of such uptake vacuoles might be much less than those of ordinary phagolysosomes (Ryser, 1967).

When it was established that the amount of RNA taken up by cells from PBS was greater than that taken up from sucrose solutions, it was postulated that the intracellular degradation of the RNA molecules was more extensive in cells incubated in PBS than in those incubated in the sucrose media. The experiments involving the reisolation and analysis of RNA from cells infected with ³H-Mengo RNA that were designed to test this premise failed to provide confirmatory data. This, naturally, was disappointing. However, the fact that no well defined peak of intact (35S) labelled viral RNA could be isolated from infected cells in

any case does not eliminate the possibility that the hypothesis is correct. The successful isolation of ^{35}S viral RNA from cells infected with intact, ^3H -labelled virus, suggests that extensive degradation did not occur during the isolation procedure. It is hard to avoid the conclusion that almost all naked, viral RNA molecules taken up by cells are rapidly broken down to small, non-infectious fragments. The absolute amount of RNA taken up by cells from either PBS or sucrose solution is extremely small, and, if degradation is as extensive as the data indicates, the detection methods used could not distinguish between the survival of 20 RNA molecules in cells incubated in sucrose and the survival of 1 molecule of RNA in cells incubated in PBS (infectious center formation in sucrose : infectious center formation in PBS = 15 - 20 : 1).

None of the working hypotheses concerning the mechanism by which DMSO exerts its stimulating effect on infectious center formation found support in the data obtained from this study. It was shown that DMSO does not enhance the uptake of RNA by cells, and it was found to stimulate, rather than inhibit, the degradation of RNA by pancreatic ribonuclease. Measurements of cell viabilities, using three different criteria of viability, failed to provide any evidence that DMSO protects cells against loss of viability in solutions of elevated osmolarity.

From the results obtained from the single cycle growth studies described in this chapter, it seems clear

that the production of progeny virus begins earlier, and the burst size is larger in cells infected with Mengo RNA in 0.6 M sucrose/PBS-10% DMSO than in cells infected in 0.6 M sucrose/PBS. These data are consistent with the proposition that the viral RNA initiates the infectious cycle earlier and more efficiently in cells infected in the presence of DMSO than in those infected in its absence, and that in the former case, a higher proportion of the invading viral genomes escape degradation at the intracellular level. It seems at least possible that DMSO may increase the number of free ribosomes available to form polyribosome structures with viral RNA (a very early if not the initial event in the infectious cycle). Existing polyribosomes are probably not disrupted by DMSO, since it was found that polyribosomes isolated from rabbit reticulocytes were not broken down during incubation with DMSO. However, DMSO may dissociate ribosomes from the endoplasmic reticulum, a possibility which has not been examined. It is also possible, that by altering the secondary structure of the viral RNA molecule, DMSO may enhance the efficiency with which the RNA can bind to already available ribosomes.

Whatever the mechanism by which DMSO stimulates infectious center formation may be, it is clear that to be effective, the compound must be present during that period when cells are incubated with the viral RNA. If

its effect is on either some aspect of cellular metabolism or on the physical parameters of some cell constituent(s), that effect must be readily reversible. When cells were incubated briefly with DMSO, washed, and incubated with RNA in 0.6 M sucrose/PBS, the number of infectious centers produced therein was found to be no greater than the number produced in control cells incubated (without pre-treatment) with RNA in 0.6 M sucrose/PBS.

The mechanism by which DEAE-D stimulates infectious center formation is not clearly defined either, although some aspects of RNA-DEAE-D-cell interaction have been well established. Despite the many, often conflicting, observations by various investigators who have examined this method of assay of infectious viral RNA, the fact that binding does occur between DEAE-D and RNA molecules is generally accepted. Maes et al. (1967) mixed various amounts of DEAE-D with 200 μ g samples of RNA, and reported that at DEAE-D:RNA ratios (w/w) in the range 1:10 to 4:10, the nucleic acid was precipitated, but that at higher DEAE-D concentrations the RNA went back into solution and a colloidal state seemed to be attained. Their observations are at odds with certain of those made during the present study. For example, it was shown (see Figure 3.7) that when DEAE-D and RNA were mixed in the ratio of 1:4 and subjected to sucrose density gradient analysis, much of the RNA sedimented to the position expected of free

viral RNA. Bachrach (1966), from data obtained from comparative RNA titrations in which DEAE-D was present either in the diluent used for making serial dilutions of FMD RNA, or in which it was added to the RNA solutions after dilution, concluded that relatively stable RNA-polycation complexes, consisting of multiple chains of each component, were formed. Although the data that this investigator published to support this conclusion was meagre, to say the least, and subject to other interpretations, the conclusion reached is in agreement with that based on the results obtained in the present study.

It is also generally accepted that the RNA-DEAE-D complex formed becomes associated with cells, and it is assumed that the RNA is taken up by the cells while still complexed with the polycation. Observations described in this chapter (see Tables 3.1 and 3.2) provide evidence that although the uptake of RNA (defined as that RNA that becomes relatively firmly cell-associated) is increased quite dramatically by the addition of DEAE-D to the cell-RNA mixture, a high proportion of the complexes are merely adsorbed to the cell membrane and elute readily therefrom. The process by which such a large, multi-stranded structure would penetrate the cell membrane is not clear. Ryser (1967) has reported that at very low concentrations, basic proteins and polyamino acids stimulate albumin uptake by pinocytosis without altering the integrity of the cell,

but that at higher polycation concentrations, the uptake is due to non-specific cellular damage (Ryser, 1967). The observations described here (see Figure 3.9) regarding the uptake of ^3H -Mengo RNA and infectious center formation over a range of DEAE-D concentrations are difficult to explain. It is possible that at low polycation concentrations (20-50 $\mu\text{g/ml}$) one obtains effective binding to but very little penetration of the cells, that at intermediate concentrations (100-500 $\mu\text{g/ml}$) minor cellular damage, sufficient to permit the complex to penetrate the cells (and to initiate viral replication) occurs, and that at the highest concentrations (1000-2000 $\mu\text{g/ml}$) cellular damage is so extensive that the cells are not capable of supporting viral replication. It is also possible that the size of the RNA-DEAE-D complex may be determined by the relative concentrations of the two components (although none of the data compiled in this study would suggest as much), and that, in some way, the size of the complex determines the efficiency with which the viral RNA can express its biological function.

Regardless of the mechanism by which RNA uptake is enhanced, the increase in uptake is not quantitatively great enough to account for the dramatic increase in the number of infectious centers formed in the presence of DEAE-D. Other factors must also be involved. Maes et al. (1967) reported that some protection against pancreatic

RNase was afforded to polio RNA by the addition of DEAE-D, although the data purporting to support this conclusion is virtually uninterpretable. These investigators further suggested that although phosphodiester linkages in the viral RNA may be split to some extent, the molecule may be so firmly held "in position" by the DEAE-D that it can still function as a messenger when it enters the cell. This suggestion is hard to take seriously in view of the observation by Berlinguet and Normand (1968) that in vitro protein synthesis is blocked by the addition of either poly-L-lysine or histones, presumably because these compounds bind to the messenger RNA so that it can no longer enter into polyribosome structures. It seems more reasonable to postulate that the RNA must be liberated from the complex before it can express its genetic information, and that the observed delay in the production of progeny virus in cells infected with RNA in the presence of DEAE-D is due to the time required for the breakdown of the complex.

These studies have provided convincing evidence in support of the premise that the limiting factor with respect to the formation of infectious centers in cell-viral RNA mixtures is not the amount of intact viral RNA present in the solution, but is, rather, one or more aspects of cell-RNA interaction,- the inefficiency with which the RNA is taken up, the extent to which the RNA is degraded at the intracellular level, the efficiency with which the RNA is

incorporated into polysomes, or, perhaps the inability of a fraction of the cell population to be productively infected. Two of the studies reported here bear on this latter possibility. It was shown that, when five successive cell samples were incubated with ^3H -Mengo RNA in the presence of DEAE-D, the same number of infectious centers were formed in each, although the amount of RNA that became cell-associated decreased progressively from sample 1 through sample 5. Perhaps the most obvious explanation of this observation is that, in each sample, every cell in the population was "infected" - but with a progressively decreasing multiplicity (of viral RNA molecules), and, that only a fixed proportion of the cells in each sample was capable of being productively infected (capable of registering as infectious centers). The second pertinent finding is that, in a fixed number of cells, there was a linear relationship between RNA concentration and the number of infectious centers formed up to a critical concentration of RNA (see Figure 3.9). A further increase in RNA concentration above that level was not accompanied by an increase in the number of infectious centers formed, and the latter never exceeded approximately 10% of the cell population. This latter phenomenon was also observed by Koch and Bishop (1968). Other data, obtained in this laboratory as part of a separate study, has shown that at least 10^2 viral RNA

molecules are taken up per cell from PBS and as many as 10^4 molecules/cell from the PBS-DEAE-D medium. Taken together, these observations suggest that only a certain proportion of the cells in an asynchronously growing population may be capable of being productively infected by naked viral RNA at any given time,- a not entirely satisfactory proposition in that virtually all cells in such populations can be infected simultaneously with an appropriate multiplicity of virus particles. This may mean merely that, in most cells, the foreign macromolecule is rapidly destroyed upon entering the cell, and data suggesting that the intracellular survival of an intact viral RNA molecule may be a rare event has been presented in this chapter. This conclusion may be emphasized by certain calculations based on the data presented in Table 3.1. Consider only the data obtained from incubating cells for 6 mins in 0.6 M sucrose/PBS containing 10% DMSO. Assuming, for the sake of simplicity, that the concentration of ^3H -Mengo RNA used was $1.7 \mu\text{g/ml}$, and knowing that the mol. wt. of Mengo RNA is 1.7×10^6 , one can calculate that one ml of incubation media contained 6×10^{11} molecules of RNA, of which 3.6×10^8 molecules (0.06%) were taken up by 5×10^6 cells. Of these 5×10^6 cells, only 41,800 were productively infected (i.e., titrated as infectious centers), from which it can be calculated that approximately 9000 molecules of RNA were taken up for every infectious center

formed. If only one intact RNA molecule is required to initiate the infectious cycle, this may mean that only one RNA molecule of each 9000 taken up escaped degradation in this particular experiment. Given these values, it is easy to understand why all attempts to re-isolate 35S viral RNA from cells infected with viral RNA (see Figure 3.6) were unsuccessful.

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